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A Quantitative Theory of Synergism and Antagonism among Diverse Inhibitors, with Special Reference to Sulfanilamide and Urethane

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Introduction

The data and theory which have recently been set forth concerning the nature of enzyme inhibitions in bacterial luminescence (Johnson, Brown, and Marsland, 1942, a, b; Brown, Johnson, and Marsland, 1942; Eyring and Magee, 1942; Johnson, Eyring, and Williams, 1942) make it possible to interpret the action of single inhibitors, such as sulfanilamide and urethane, respectively, in relation both to concentration and to temperature. This theory has since been applied with success to a variety of typical narcotics, including monacetin, salicylamide, evipal, amytal, chlorotone, and others (McElroy, 1943).

The present paper deals with the effect on a given process of two different inhibitors, simultaneously present. Data from experiments with the luminescent system, both in intact bacterial cells and in extracts, illustrate all three possible results of mixing two inhibitors, in comparison with the effect of each separately, namely: *synergism*,[1] in which the inhibition of the two together may be considerably greater than that of either alone; *antagonism*, in which the inhibition caused by the mixture is less than that of one, and sometimes less than either alone; and practically *no effect*, in which the inhibition caused by the mixture is the same as that of either one alone. Under the appropriate conditions of con-

[1] Although widely used, the terms "synergism" and "antagonism" have no generally accepted, quantitative meanings. To use them to describe deviations from independent action of two drugs involves having a criterion for independence of action. Equations (9), (11), (14), and (16) provide this criterion if both drugs act at the same site, but other criteria are required for other types of action. Because of this complication we have adopted the definitions given above.

centration and of temperature, most of the possible effects can be illustrated with sulfanilamide and urethane, which are known to act directly on the luminescent system in *Cypridina* extracts (Johnson and Chase, 1942) and very likely do also in living bacteria.

The theoretical formulations which are derived in this paper and which are illustrated by the data presented herewith are all implicit in the theory described earlier, as are also, certain predictions concerning changes in the apparent optimum temperature of the reaction. The latter have been formulated (Johnson and Eyring, 1943) but the complete derivations and the supporting data will be given at a later date.

Theoretical Basis

The same notation employed in the previous papers will be used in this, as follows:

I_1 = luminescence intensity without added inhibitor. For the reasons previously described in detail, luminescence intensity is assumed proportional to the reaction velocity in the luminescent oxidation (equation 1).

I_2 = luminescence intensity after inhibitor addition.

b = proportionality constant, allowing for the units of measurement.

k_2 = rate constant.

LH_2 = luciferin, the substrate in the luminescent oxidation.

A_o = total amount of luciferase, the enzyme concerned in light emission.

A_n = active, or native luciferase, uncombined with inhibitor, capable of uniting with luciferin in the light emitting reaction.

A_d = inactive, reversibly denatured luciferase, in equilibrium K_1 and A_n. This equilibrium is characterized by a high heat of reaction, amounting to 60,000 to 90,000 calories.

(X) = molar concentration of inhibitor X.

r = number of molecules of inhibitor combining with one molecule of A_n.

s = number of molecules of inhibitor combining with one molecule of A_d, or combining with A, without specifying A_n or A_d. In addition to the above, the following will also be used in this paper:

I_x = intensity of luminescence in the presence of a given concentration of X, in which X is a Type I inhibitor, such as sulfanilamide.

I_y = Ditto, with a different Type I inhibitor, Y, such as *p*-aminobenzoic acid.

I_u = Ditto, in which U is a Type II inhibitor, such as urethane.

I_w = Ditto, for a second Type II inhibitor, w, for example, butyl alcohol.

I_{x+u}, I_{x+y}, I_{x+w}, I_{w+y} represent the luminescence intensity in the presence of given concentration of the inhibitors, indicated by subscripts, simultaneously present. Where more than one inhibitor of the same type is involved, a prime (′) is used to designate the equilibrium constant of the second inhibitor.

The existence of a reversible temperature inactivation of the enzyme, discussed at length in the previous papers, is assumed. It has provided the key for a satisfactory interpretation of the action of urethane and sulfanilamide acting separately on luminescence, and it is basic in the theory of the effects of mixed inhibitors considered herewith. In general, any added inhibitor which enters into a reversible combination with the enzyme may combine to form one or more products. These have been designated as forming the following types: Type I, the inhibitor combines indiscriminately with A_n and A_d; Type II, the inhibitor combines only with A_d; and type III, it combines only with A_n. Thus far it has not been necessary, in the interpretation of the data, to consider the possible intermediate types, where the inhibitor combines with both A_n and A_d but with different equilibrium constants.

It is now possible to state somewhat more precisely the character of the sulfanilamide and urethane equilibria. Moreover, since Types II and III are physically indistinguishable the diagrammatic representation given previously (Johnson, Eyring, and Williams, 1942) as a formal basis for the three types can now be replaced by two, more explicit diagrams to represent the Type I and Type II inhibitions respectively. Thus, in Type I the combination of the inhibitor with the enzyme gives rise to two distinct products, A_nX_r and A_dX_r, according to whether it is the native, active, or the denatured, inactive forms of the protein. In Type II there is only one product, AU and it is merely a matter of point of view whether this product is formed through the two equilibria, K_1 and K_3, or a single equilibrium with the constant K_1K_3. These conclusions follow from the agreement of theoretical derivations on the basis of these diagrams with data from experiments. Because the equilibrium constant K_1, for $A_n \rightleftharpoons A_d$ is the same as for $A_nX_r \rightleftharpoons A_dX_r$, in Type I, different parts of the luciferase molecule are involved in the addition of the inhibitor and in the reversible denaturation by temperature. On the other hand, in Type II the inhibitor combines at sites made available by the heat denaturation, and consequently there is only a single product, whether the inhibitor adds to A_n or to A_d.

As in the previous studies, for the reasons stated, the intensity of luminescence is assumed proportional to the rate constant, which has been consistently designated as k_2, times the luciferase concentration times the luciferin concentration:

$$I = bk_2(LH_2)(A_n) \tag{1}$$

The inhibitors, urethane and sulfanilamide, as well as certain others (Johnson and Chase, 1942) are known to act directly on the considerably purified, extracted light-emitting enzyme-substrate system of *Cypridina*. The other inhibitors considered in this paper very likely do also, along with a variety of narcotics (Taylor, 1934). In the discussion below, it is assumed that the inhibitors act by combining with the enzyme, rather than the substrate. The various possible cases will now be considered.

1. Single inhibitors

a. Luminescence intensity without added inhibitor

Total luciferase $= (A_o) = (A_n) + (A_d)$. Since $(\frac{A_d}{A_n}) = K_1$, then $(A_n) = \frac{A_o}{1 + K_1}$ and therefore,

$$I_1 = \frac{bk_2(LH_2)(A_o)}{1 + K_1} \tag{2}$$

b. With a single inhibitor of Type I,

$$(A_o) = (A_n) + (A_d) + (A_n X_r) + (A_d X_r) \tag{3}$$

But since $A_n + rX \rightleftharpoons A_n X_r$, and $A_d + rX \rightleftharpoons A_d X_r$, we have

$$(A_n X_r) = K_2(A_n)(X)^r, \quad \text{and} \quad (A_d X_r) = K_2(A_d)(X)^r = K_2 K_1(A_n)(X)^r.$$

Hence, $(A_o) = (A_n) + K_1(A_n) + K_2(A_n)(X)^r + K_1 K_2(A_n)(X)^r$, or,

$$(A_n) = \frac{(A_o)}{(1 + K_1)(1 + K_2(X)^r)} \tag{4}$$

c. For a single inhibitor of Type II we have,

$$(A_o) = (A_n) + (A_d) + (AU_s), \quad \text{and since} \quad \frac{(AU_s)}{(A_n)(U)^s} = K_1 K_3\,,$$

we may write $(A_o) = (A_n) + K_1(A_n) + K_1 K_3(A_n)(U)^s$, or,

$$(A_n) = \frac{(A_o)}{1 + K_1 + K_1 K_3(U)^s} \tag{5}$$

2. Mixtures of inhibitors that do not combine with each other

In this group we will assume that the inhibitors do not combine with each other. We then have four possibilities, as follows (d, e, f, g):

d. Two type I inhibitors, X and Y, one molecule of either one being sufficient to inhibit the activity of one luciferase molecule.

$(A_o) = (A_n) + (A_d) + (A_nX) + (A_dX) + (A_nY) + (A_dY)$, or, as before,

$$(A_o) = (A_n) + K_1(A_n) + K_2(A_n)(X) + K_1K_2(A_n)(X) + K_2'(A_n)(Y) + K_1K_2'(A_n)(Y),$$ whence,

$$(A_n) = \frac{(A_o)}{(1 + K_1)(1 + K_2(X) + K_2'(Y))} \tag{6}$$

Substituting in (1) we obtain

$$I_{x+y} = bk_2(LH_2)(A_n) = \frac{bk_2(LH_2)(A_o)}{(1 + K_1)(1 + K_2(X) + K_2'(Y))} \tag{7}$$

Dividing equation (2) by equation (7) and simplifying,

$$\frac{I_1}{I_{x+y}} = 1 + K_2(X) + K_2'(Y) \tag{8}$$

Since this formula applies to all concentrations of X and Y, including the cases where either of the two is 0, we have

$$\left(\frac{I_1}{I_{x+y}} - 1\right) = \left(\frac{I_1}{I_x} - 1\right) + \left(\frac{I_1}{I_y} - 1\right) \tag{9}$$

e. If instead of one molecule of inhibitor, r molecules of either X or Y are required to inactivate each enzyme molecule, we can define an effective concentration of X.

$$(X)_{\text{effective}} = \left(X + \left(\frac{K_y}{K_x}\right)^{\frac{1}{r}} Y\right),$$ and at once obtain, as before,

$$\left(\frac{I_1}{I_{x+y}} - 1\right) = K_x\left(X + \left(\frac{K_y}{K_x}\right)^{\frac{1}{r}} Y\right)^r \tag{10}$$

whence,

$$\left(\frac{I_1}{I_{x+y}} - 1\right)^{\frac{1}{r}} = \left(\frac{I_1}{I_x} - 1\right)^{\frac{1}{r}} + \left(\frac{I_1}{I_y} - 1\right)^{\frac{1}{r}} \tag{11}$$

In cases d and e above, it is assumed that the molecules of X and of Y act interchangeably. The same formulas would, therefore, hold for two concentrations of the same inhibitor, X or Y. The inhibitions of two concentrations are additive according to these formulations, which

thus provide a ready means of testing, with experimental data, whether a single system is affected by a given inhibitor, in this manner.

f. Type I plus Type II, acting independently of each other on the same enzyme.

$$(A_o) = (A_n) + (A_d) + (A_nX_r) + (A_dX_r) + (AU_s) + (AU_sX_r)$$

$$(A_o) = (A_n) + (A_n)K_1 + K_2(A_n)(X)^r + K_1K_2(A_n)(X)^r + K_1K_3(A_n)(U)^s + K_2K_1K_3(A_n)(U)^s(X)$$

$$(A_n) = \frac{(A_o)}{1 + K_1 + (1 + K_1)K_2(X)^r + K_1K_3(U)^s(1 + K_2(X)^r)} \tag{12}$$

$$\frac{I_1}{I_{x+u}} = 1 + K_2(X)^r + \frac{K_1K_3(U)^s}{1 + K_1} + \frac{K_2K_1K_3(U)^s(X)^r}{1 + K_1} \tag{13}$$

$$\frac{I_1}{I_{x+u}} = (1 + K_2(X)^r)\left(1 + \frac{K_1K_3(U)^s}{1 + K_1}\right) = \frac{I_1}{I_x}\cdot\frac{I_1}{I_u}, \text{ or, } \frac{I_x}{I_{x+u}} = \frac{I_1}{I_u} \tag{14}$$

g. Two Type II inhibitors, U and W, respectively, acting interchangeably, where s molecules are required in reversibly denaturing one enzyme molecule. In this case, again, there is an effective concentration,

$$U_{\text{effective}} = \left((U) + \left(\frac{K_w}{K_u}\right)^{\frac{1}{s}}(W)\right) \tag{15}$$

$$\left(\frac{I_1}{I_{u+w}} - 1\right)\left(1 + \frac{1}{K_1}\right) = K_u(U_{\text{effective}})^s = K_u\left((U) + \left(\frac{K_w}{K_u}\right)^{\frac{1}{s}}(W)\right)^s$$

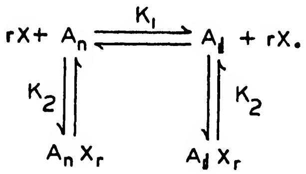
$$\left[\left(\frac{I_1}{I_{u+w}} - 1\right)\left(1 + \frac{1}{K_1}\right)\right]^{\frac{1}{s}} = (K_u)^{\frac{1}{s}}(U) + (K_w)^{\frac{1}{s}}(W)$$

$$= \left[\left(\frac{I_1}{I_u} - 1\right)\left(1 + \frac{1}{K_1}\right)\right]^{\frac{1}{s}} + \left[\left(\frac{I_1}{I_w} - 1\right)\left(1 + \frac{1}{K_1}\right)\right]^{\frac{1}{s}} \tag{16}$$

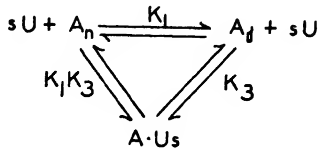
3. Mixtures of inhibitors that combine with each other

From general considerations, as well as from the data available thus far, it would not appear likely that one simple Type I inhibitor would combine with another simple Type I inhibitor. We have not encountered a case where two Type II inhibitors combine with each other. In contrast, however, a Type I inhibitor may be expected to combine with a Type II inhibitor. The diagram Type I plus Type II illustrates the several equilibria which would then ensue.

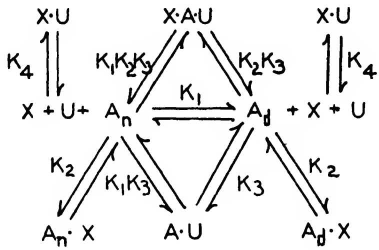
TYPE I



TYPE II



TYPE I plus TYPE II



In this case it is convenient to consider first the simplest conditions namely, when the two substances combine in a ratio of 1:1, perhaps forming a dipole compound; when they do not interfere with each other in combining with the enzyme; and when the Type II is present in considerable excess. The following formulations are based on these three assumed conditions.

h. Type I plus Type II, with Type II in excess.

$$\begin{aligned}(A_o) &= (A_n) + (A_d) + (A_nX_r) + (A_dX_r) + (AU_s) + (AX_rU_s)\\ &= (A_n)[1 + K_1 + (X)^r K_2 + (X)^r K_2 K_1 + K_1K_3(U)^s + K_1K_3K_2(X)^r(U)^s]\end{aligned}$$

$$(A_n) = \frac{(A_o)}{[1 + K_1 + K_1K_3(U)^s]\,[1 + K_2(X)^r]} \tag{17}$$

$$\frac{I_1}{I_{x+u}} = \left(1 + \frac{K_1K_3(U)^s}{1 + K_1}\right)(1 + K_2(X)^r) \tag{18}$$

From the assumed conditions regarding the equilibrium, $X + U \rightleftharpoons XU$, we have

$$(X_o) = (X) + (XU), \quad \text{and} \quad \frac{(XU)}{(X)(U)} = K_4$$

Thus,

$$(X_o) = (X)(1 + K_4(U_o)), \text{ or } (X) = \frac{(X_o)}{1 + K_4(U)} \tag{19}$$

Substituting in equation (18),

$$\frac{I_1}{I_{x+u}} = \left(1 + \frac{K_1K_3(U)^s}{1 + K_1}\right)\left(1 + \frac{K_2(X)^r}{(1 + K_4(U_o))^r}\right)$$

$$= \frac{I_1}{I_u}\left(1 + \frac{I_1 - I_{x_o}}{I_{x_o}(I + K_4(U))^r}\right)$$

$$\left(\frac{I_u}{I_{x+u}} - 1\right)\left(\frac{I_x}{I_1 - I_{x_o}}\right) = \frac{1}{(1 + K_4(U_o))^r}\Big)$$

$$\left[\left(\frac{I_1 - I_{x_o}}{I_u - I_{x+u}}\right)\left(\frac{I_{x+y}}{I_{x_o}}\right)\right]^{\frac{1}{r}} - 1 = K_4(U_o) \tag{20}$$

In equation (20), if $r = 1$, and $K_4 = 0$, the expression simplifies as follows:

$$\frac{I_1 - I_x}{I_u - I_{x+u}} = \frac{I_x}{I_{x+u}}$$

$$I_1 I_{x+y} - I_x I_{x+u} = I_u I_x - I_{x+u} I_x$$

$$\frac{I_1}{I_x} = \frac{I_u}{I_{x+u}}, \text{ or, } \frac{I_1 I_{x+u}}{I_x I_u} = 1,$$

which is the same as equation (14).

i. Type I plus Type II, when the two are added in nearly the same amounts. In this case, the equation becomes complicated because of the changing concentrations of both X_o and U_o as they combine with the enzyme and with each other. The solution is as follows:

$$\frac{(XU)}{(X)(U)} = K_4$$

The expressions for X and for U are:

$$(X_o) = (X) + (XU) = (X) + K_4(X)[(X) - (X_o) + (U_o)]$$

$$= (X) + K_4(X)^2 + K_4(X)[(U_o) - (X_o)]$$

$$K_4(X)^2 + [K_4((U_o) - (X_o)) + 1]\,(X) - (X_o) = 0$$

$$X = \frac{K_4[(X_o) - (U_o)] - 1 \pm \sqrt{[K_4((U_o) - (X_o)) + 1]^2 + 4K_4(X_o)}}{2K_4} \tag{21}$$

$$(U_o) = (U) + (XU)$$

$$(U) = (U_o) - (X_o) + (X)$$

$$(U) = (U_o) - (X_o) + \frac{K_4[(X_o) - (U_o)] - 1 \pm \sqrt{[K_4((U_o) - (X_o)) + 1]^2 + 4K_4(X_o)}}{2K_4}$$

Hence,

$$\frac{I_1}{I_{x+u}} = \left(1 + \frac{K_1 K_3}{1 + K_1}\left\{(U_o) - (X_o) + K_4[(X_o) - (U_o)] - 1 \pm \sqrt{(K_4[(U_o) - (X_o)] + 1)^2 + 4K_4(X_o)}\right\}^s\right)$$

$$\cdot\left(1 + K_2\left\{\frac{K_4[(X_o) = (U_o)] - 1 \pm \sqrt{(K_4[(U_o) - (X_o)] + 1)^2 + 4K_4(X_o)}}{2K_4}\right\}^r\right) \quad (22)$$

In applying equation (22) to data from experiments, the calculation becomes laborious. Fortunately, most of the mixtures of inhibitors used in the present study have not necessitated the use of this equation. Chloroform and sulfanilamide apparently illustrate such a case, but the complete calculations in this connection have not been carried out.

Experimental Methods

Since the methods used for the preparation of bacterial suspensions, measurement of luminescence, et cetera have already been described at length (Brown, Johnson, and Marsland, 1942; Johnson, Eyring, and Williams, 1942) there is little need to enlarge upon them again. The cells of brightly luminous agar cultures were emulsified in phosphate buffered sodium chloride solution. Corresponding portions of a given suspension, with and without the addition of a given inhibitor or mixture of inhibitors, were placed in tubes of selected uniformity and the luminescence measured at constant temperature, or in relation to temperature, in a water bath. With non-volatile inhibitors, the cells were aerated continuously with a fine stream of oxygen. With others, such as ether and acetone, a small volume of the bacterial suspension, not more than 10% of the final volume, was added to a solution previously saturated with oxygen. The inhibitor itself was added to salt solutions saturated with oxygen, and dilutions made with the same medium. ' A cork stopper was then placed in the tube, and aeration of the cells accomplished by occasional, vigorous shaking.

In experiments involving the use of washed cells, the procedure of Johnson, van Schouwenburg, and van der Burg (1939) for the preparation of the cell suspension was followed.

Application of the Theory to Data from Experiments

Sulfanilamide, p-aminobenzoic acid, and urethane

The relation of luminescence intensity to temperature is shown in Fig. 1 for two unrelated species of bacteria, in the presence of sulfanilamide, urethane, and *p*-aminobenzoic acid (PAB) separately, as well as mixtures of urethane plus sulfanilamide, and inhibitory concentrations of PAB plus sulfanilamide. The relation of the sulfanilamide and PAB

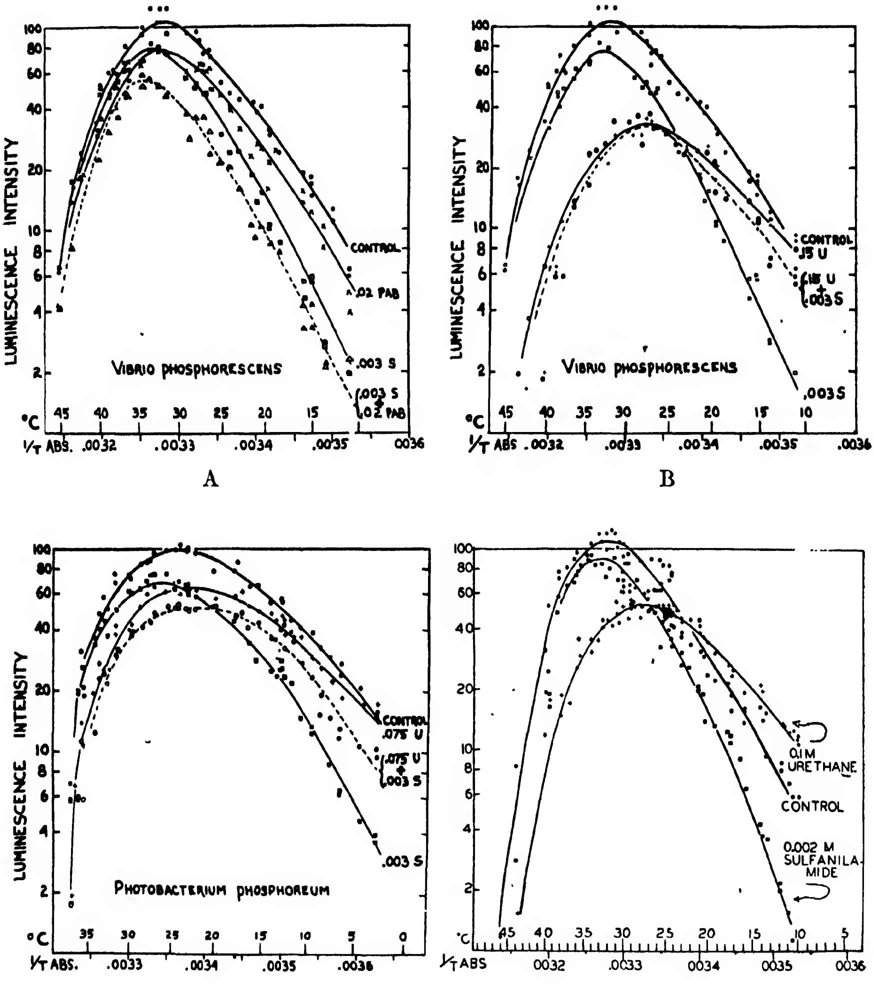


Fig. 1

The Relation of Luminescence Intensity to Temperature With and Without Added Inhibitors, and Mixtures of Inhibitors, as Indicated at the Right of the Respective Curves

Abscissae: reciprocal of the absolute temperature; ordinates: luminescence intensity, calculated on the basis that the maximum equals 100, and represented on a logarithmic scale. A, B, and D, *Vibrio phosphorescens*; C, *Photobacterium phosphoreum*.

inhibitions of luminescence to temperature is in accordance with expectations from their earlier analysis, indicating Type I inhibitors. The per cent inhibition is greater at low temperatures. The apparent optimum temperature of luminescence is slightly greater. The action of urethane, on the other hand, results in a greater inhibition at the higher than the lower temperatures, and the presence of this substance, acting as a Type II inhibitor, causes the apparent optimum temperature of luminescence to decrease.

Mixtures of the inhibitors give some interesting results. When inhibitory concentrations of two different Type I inhibitors are simultaneously present, a stronger inhibition takes place than with either one of the substances, in the same concentration, alone. This relation holds throughout the entire temperature range. The application of

TABLE I

The Inhibitory Action of 0.003 M Sulfanilamide and 0.02 M p-Aminobenzoic (PAB) Acid, each Separately and when Mixed Together, on the Luminescence of Vibrio phosphorescens at Different Temperatures

(Notation as given in the text)

Temp. °C.	I_1	I_S	I_{PAB}	I_{S+PAB}	$\left(\frac{I_1}{I_S}-1\right)$	$\left(\frac{I_1}{I_{PAB}}-1\right)$	Total	$\left(\frac{I_1}{I_{S+PAB}}-1\right)$
15	15.5	5.35	11.2	3.6	1.9	0.38	2.28	3.30
20	34.5	15.0	25.0	9.8	1.3	0.38	1.68	2.52
25	63.0	35.0	47.0	22.0	0.8	0.34	1.14	1.86
30	102.0	65.0	72.0	43.0	0.57	0.42	0.99	1.37
35	105.0	77.5	77.0	52.5	0.35	0.36	0.71	1.00

equation (9) yields results that are in fair agreement with expectations (Table I). The discrepancy is not more than might be accounted for on the basis of a slight difference in pH between the sulfanilamide and PAB solutions.

In contrast to the results with PAB, the addition of urethane to sulfanilamide leads to a result which is either antagonistic or synergistic with respect to the sulfanilamide inhibition, purely according to temperature (Fig. 1, B and C). At low temperatures, where a given concentration of urethane alone has little effect, it may actually counteract a strong sulfanilamide inhibition. At low temperatures, the addition of urethane to a sulfanilamide-inhibited suspension may result in an increase of luminescence intensity amounting to several hundred per cent. At higher temperatures, urethane exerts its own inhibition by promoting reversible temperature inactivation of the enzyme, and

may thus considerably increase the extent of the inhibition resulting from sulfanilamide alone. It should be emphasized that this apparent reversal in the effect obtained by adding urethane to a sulfanilamide-inhibited suspension may be brought about purely by a change in temperature. Since the urethane is present in considerable excess, and since the two substances are known to act directly on the light emitting system, there appears to be only one interpretation for the results indicated in Fig. 1, B and C, namely, that the two substances not only combine with the enzyme, but also with each other. If this is the case,

TABLE II

The Inhibitory Effect of 0.003 M Sulfanilamide and 0.15 M Urethane, Separately and Mixed, on the Luminescence of Vibrio phosphorescens at Various Temperatures; similarly, 0.003 M Sulfanilamide and 0.075 M Urethane in relation to Photobacterium phosphoreum

Values for K_4 according to equation (20)

Temp. °C.	I_1	I_S	I_U	I_{S+U}	$\left[\left(\frac{I_1 - I_S}{I_U - I_{S+U}}\right)\left(\frac{I_{S+U}}{I_S}\right) - 1\right]$	K_4
			Vibrio phosphorescens			
15	15.8	4.15	12.2	9.9	11.05	73.7
20	34.0	13.3	21.0	18.0	8.30	55.3
25	62.0	34.5	30.5	29.0	14.40	96.0
30	100.0	65.5	30.7	29.5	12.05	80.4
35	91.0	68.0	19.5	17.0	2.82	18.8
			Photobacterium phosphoreum			
5	20.5	8.3	18.3	12.0	1.81	24.1
10	38.0	14.5	31.5	25.0	5.22	69.6
15	61.0	29.5	48.0	40.0	4.35	58.0
20	88.0	50.0	61.5	50.8	2.61	34.8
25	99.0	67.0	62.0	50.0	1.00	13.3
Average value for K_4						52.4

it should be possible to calculate the equilibrium constant for this loose, probably physical combination, or mutual adsorption, by the use of equation (20). Table II gives the results of the calculation, and indicates fairly consistent constants at all temperatures where the data are sufficiently accurate to provide a reliable basis for the calculation. The accuracy needed is extraordinarily great, because the equation involved is extremely sensitive to the least experimental error. Thus, the constants given in Table II for K_4, calculated from data on luminescence intensity of two different organisms, a variety of temperatures, and two different concentrations of urethane vary scarcely more than might be expected from small experimental errors in the measurement

of luminescence. Assuming that the true value of K_4 is the average of those in Table II, it is possible to predict with considerable accuracy the intensity of luminescence in the presence of various concentrations and proportions of the two inhibitors, from the effects of the two separately. The error of the predictions is greatest at the very low and at the very high concentrations, *i.e.*, less than 15% or more than 85% inhibition, respectively. The former discrepancy may arise from the fact that the true concentration of inhibitor, at the enzyme, is ap-

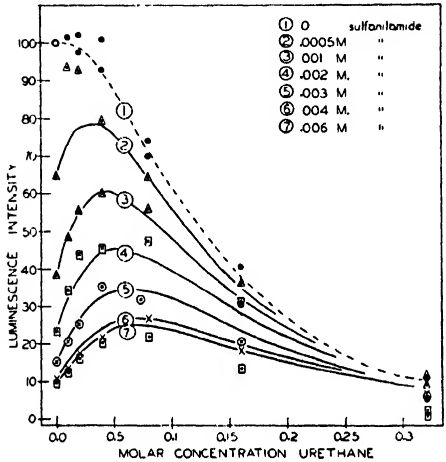


FIG. 2

Luminescence Intensity of *P. phosphoreum* at 5°C., in Relation to Various Concentrations of Sulfanilamide Alone, Urethane Alone (Dotted Line), and Mixtures of the Two in Various Proportions and Concentrations

The continuous lines are theoretically predicted curves, on the basis of the effects of the inhibitors separately, and on the assumption that sulfanilamide forms a reversible combination with the urethane, in an equilibrium whose constant is 50. The points are the actually observed, experimental values. At all except the very high and the very low concentrations of inhibitors, the agreement between theoretically predicted and the observed values is strikingly good.

preciably different from the concentration added, probably because it combines with more than one substance in the cell. The latter discrepancy might arise either because of the partially irreversible effect of high concentrations of inhibitor, increasing with time and concentration, or from a possible additive effect on more than one reaction concerned in the process of luminescence. In spite of these minor differences between the theoretically predicted and the experimentally observed values, the agreement is strikingly good (Fig. 2).

The results described above suggest that the stimulation of luminescence by addition of low concentrations of urethane alone, not only to bacteria (Taylor, 1934; van Schouwenburg, 1938) but also to unpurified extracts containing the luciferin-luciferase system of *Cypridina* (Taylor, 1934) may well result from the combination of the urethane with a substance which is already present, and which is inhibiting the rate of the reaction in somewhat the same manner as sulfanilamide does. In fact, certain experiments of the present study have substantiated the observation that small concentrations of urethane may stimulate luminescence. In view of the foregoing, one might expect such a stimulation, in relation to temperature, to resemble the urethane antagonism of sulfanilamide. Fig. 1, D, presents data from an experiment which bears out this supposition. The stimulation caused by urethane is apparent only at low temperatures, and changes to an inhibition at the higher temperatures. The experiment is important in suggesting a basis for the stimulating effects of low concentrations of poisons and narcotics in general, so familiar in pharmacology and various types of biological phenomena. This interpretation might be expected to hold in all cases where combination of an added inhibitor takes place with another substance which is normally or already present, exerting a retarding influence on the reaction in question. Other factors would also be involved, of course, where more than one system is appreciably affected, e.g., stimulation might occur by inhibition of a competing reaction. The effect of CN on luminescence at low oxygen tensions (van Schouwenburg, 1938) illustrates such a case.

Sulfanilamide plus other Type II inhibitors

The foregoing results provide a substantial basis for believing that other Type II inhibitors, including various members of the "lipoid soluble" group of narcotics, should indicate an antagonism of the sulfanilamide inhibition, at the proper temperature and concentration. The exact concentrations and temperatures, of course, cannot be predicted in advance on purely theoretical considerations, since there is no way of estimating the necessary equilibrium constants for the combinations between inhibitors and enzyme, as well as with each other. The qualitative result that might be anticipated, however, may be readily demonstrated.

Evidence from the action of hydrostatic pressure (Johnson, Brown, and Marsland, 1942) indicates that ethyl alcohol, ether, and chloroform

act as Type II inhibitors on luminescence. Certain similarities in properties at once suggest that other substances, including acetone, and alcohols other than ethyl, should also act in this manner. Although the complete data in regard to temperature and concentration, necessary to establish fully the nature of the inhibition, have been worked out only

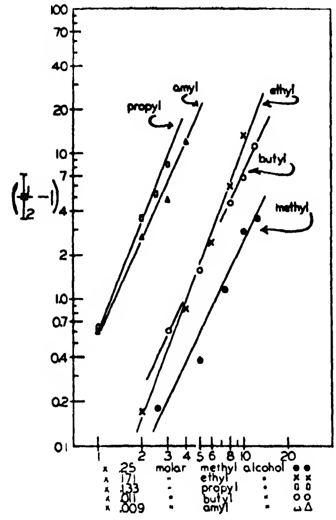


FIG. 3

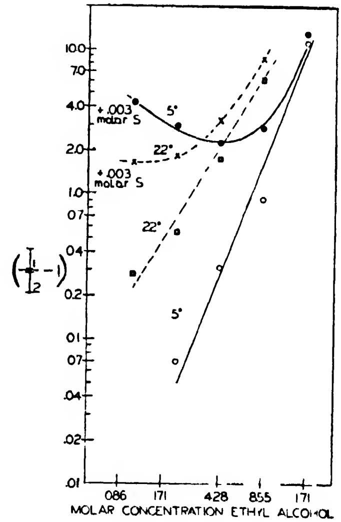


FIG. 4

FIG. 3

The Relation between the Inhibition of Luminescence and Concentration of Alcohols, Plotted as the Logarithm of $\left(\frac{I_1}{I_2} - 1\right)$ against the Logarithm of the Concentration

Temperatures of the experiments were as follows: methyl alcohol, 12.4°C.; ethyl alcohol, 13.7°C.; propyl, 11.5°C.; butyl, 11.5°C.; amyl, 12.4°C.

FIG. 4

The Inhibition of Luminescence in Relation to Concentration of Alcohol for Suspensions of Bacteria With and Without 0.003 M Sulfanilamide also Present, at 5 and 22°C., respectively

Log-log scale

for urethane and sulfanilamide, respectively, the results below leave little doubt as to the correctness of the suppositions.

The effect of various concentrations of several members of a homologous series of alcohols, alone, is shown in Fig. 3, plotted in accordance with equation (23) of Johnson, Eyring, and Williams (1942). The ab-

solute concentrations needed for a given inhibition become less, as one would expect, with decrease in water-solubility of the compound, and the accompanying increase in the tendency for it to seek out the hydrocarbon groups in proteins. It is clear, however, that the resulting straight lines have a fairly steep slope, varying between 2 and 3, and thus indicating that the ratio of alcohol molecules combining with each enzyme molecule is between 2 and 3. In these respects, the alcohols resemble urethane in action. When mixed with sulfanilamide, a similar plot of the resulting data gives quite a different picture.

In Fig. 4 the action of ethyl alcohol, alone, and in the presence of a constant amount of added sulfanilamide, 0.003 *M*, is shown for two temperatures, 5° and 22°C., respectively, plotted in the same manner as for the alcohols alone, in the preceding figure. At either temperature, a straight line of considerable slope results for alcohol, by itself, as expected. In the presence of a constant amount of sulfanilamide, which alone causes a considerable inhibition at low temperatures, the first apparent result of adding ethyl alcohol is a decrease in the amount of inhibition. This indicates that alcohol probably is acting just like urethane, and by combining with the sulfanilamide decreases the effective concentration of the latter substance. High concentrations of alcohol, though still combining with the sulfanilamide according to the same equilibrium constant as before, now give rise to a greater inhibition than with sulfanilamide alone. The same result might be achieved simply by increasing the temperature, for although a rise in temperature decreases the sulfanilamide inhibition, the temperature coefficient for the action of alcohol is evidently much greater.

In Fig. 5, the effect of increasing concentrations of sulfanilamide alone, and in the presence of given concentrations of butyl alcohol, is plotted in the same manner as before. The straight line, with slope of approximately one, for sulfanilamide alone is in marked contrast to the results obtained with mixtures. With sufficient butyl alcohol to bring about initially a considerable inhibition, sulfanilamide can be added in quite appreciable concentrations without causing a further increase in inhibition. When the concentration of sulfanilamide is sufficient to cause more inhibition than that of the alcohol alone, the effect of the latter is substantially additive. These results are essentially the same as those obtained with ethyl alcohol, discussed above.

It should be pointed out, in connection with these observations, that the data reported earlier by Johnson and Chase (1942) concerning the

inhibition of luminescence intensity of the partially purified, extracted luciferin-luciferase system of *Cypridina* were obtained with substrate solutions prepared in butyl alcohol. The actual, final concentration of

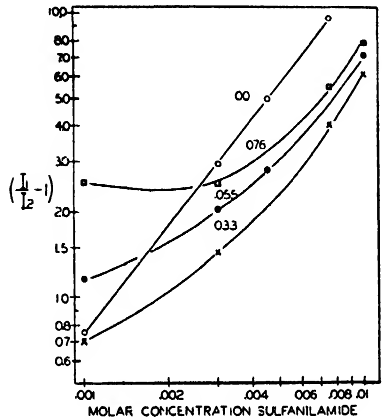


FIG. 5

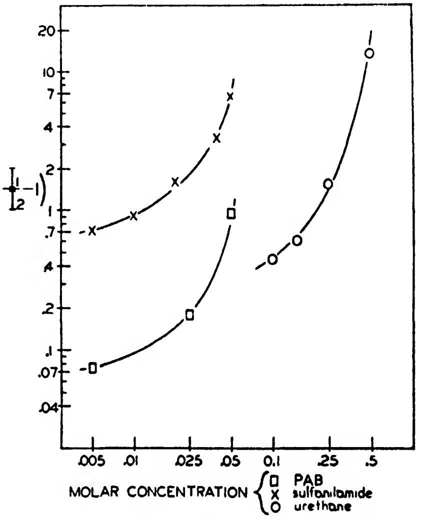


FIG. 6

FIG. 5

The Inhibition of Luminescence, at 10°C., in Relation to Concentration of Sulfanilamide Alone, and the Concentrations of Butyl Alcohol Indicated Just Above the Respective Curves
Log-log scale

FIG. 6

Relation between Concentration and Inhibition of the Velocity of the Luminescent Oxidation in Partially Purified Extracts of Cypridina, from the Data of Johnson and Chase (1942) Concerning the Effects of *p*-Amino-benzoic Acid, Sulfanilamide, and Urethane

The luciferin solution contained a final concentration of approximately 0.05 *M* butyl alcohol, at a temperature probably high enough for this substance to affect the rate of the reaction. Compare with Figs. 5 and 7, showing how the straight line relation expected for this plot is altered by the presence of butyl alcohol, in bacterial luminescence.

butyl alcohol in the reaction mixture generally used amounted to 0.5%, or approximately 0.05 *M*, and the temperature of the experiments was approximately 27°C. When the data in Table I of Johnson and Chase (1942), are plotted in the same manner as above, curves similar to present

curves, obtained with bacteria, for mixtures of sulfanilamide and alcohols are obtained, as shown in Fig. 6. Thus, it is clear that the amount of butyl alcohol in the solvent, at this temperature, influences the action not only of sulfanilamide, but also of PAB and of urethane. At a lower temperature, this concentration of butyl alcohol alone might easily have

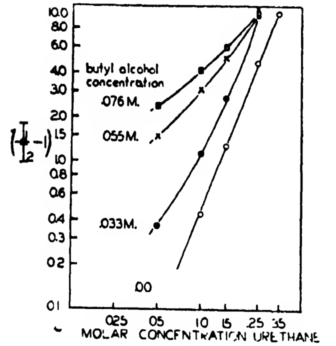


FIG. 7

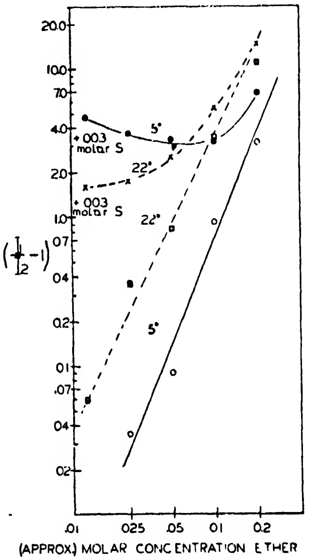


FIG. 8

FIG. 7

The Inhibition of Bacterial Luminescence in Relation to Concentration of Urethane Alone, and Urethane Plus Constant Concentrations of Butyl Alcohol Indicated at the Left of the Respective Curves

FIG. 8

The Inhibition of Luminescence in Relation to Concentration of Ether, With and Without a Constant Concentration of 0.003 M Sulfanilamide, at 5° and 22°C., Respectively

no effect on the course of the reaction. Fig. 6 shows that mixtures of urethane with butyl alcohol, comprising two Type II inhibitors, similarly disturb the straight line relation of the plot of log $(I_1/I_3 - 1)$ against log. concentration. There is no evidence, however, that a combination takes place between the two inhibitors. In Fig. 7, the results of adding various concentrations of butyl alcohol to urethane is shown

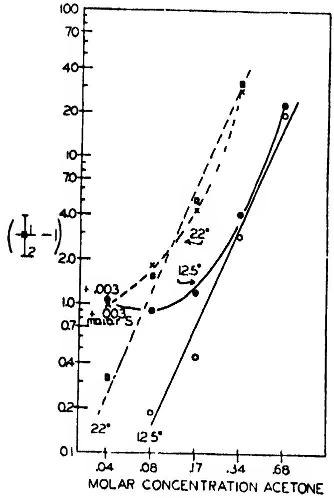


FIG. 9

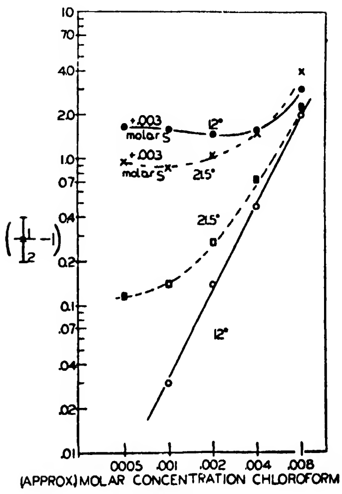


FIG. 10

FIG. 9

The Inhibition of Luminescence in Relation to Concentration of Acetone, With and Without a Constant Concentration of 0.003 M Sulfanilamide, at 12.5° and 22°C., Respectively

FIG. 10

The Inhibition of Luminescence in Relation to Concentration of Chloroform With and Without a Constant Concentration of 0.003 M Sulfanilamide, at 12° and 21.5°C., Respectively

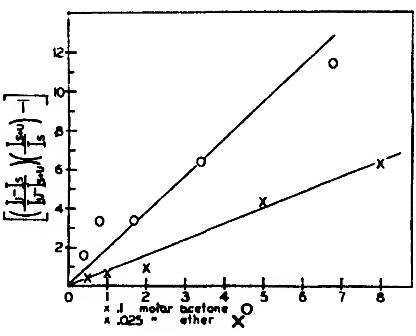


FIG. 11

The Equilibrium Constants, K_4 (Slopes of the Respective Lines) for the Reversible Combination of Sulfanilamide With Ether and Acetone, Respectively, Calculated According to Equation (20)

for bacterial luminescence. The addition of the former always increases the inhibition caused by the latter alone.

The results obtained with ether, acetone, and chloroform are in accord with expectations, except that with chloroform alone, a straight line results only at the lower temperature. This probably indicates that a certain amount of irreversible effect on luminescence occurs at the higher temperature, which leads to a greater inhibition for a given concentration (Fig. 10). Chloroform is evidently much more potent in its action, and smaller concentrations are needed. In fact, the concentrations employed are so nearly within the same range as the sulfanilamide, that the complex equation (22) would have to be applied to calculate the equilibrium constant for the chloroform-sulfanilamide combination. This we have not carried out, because of the time and work which would be required. The equilibrium constants for the acetone and the ether combinations with sulfanilamide, however, can be easily determined with the aid of equation (20). They are represented by the slopes of the lines in Fig. 11. It should be noted that this figure is not plotted on a logarithmic scale, and in spite of the sensitivity of the formula to experimental error, the points are obviously along straight lines. These lines must of necessity pass through the origin, unless there is an unknown inhibiting agent already present, thereby changing the true position of the origin on the abscissa.

Solubility of Mixtures

A reversible combination between two substances might be expected to become evident in the solubility of these substances. The solubility of the product cannot be predicted *a priori*, but except in the most extraordinary circumstances compound formation will alter the apparent solubility of the individual components. The combination itself might be a true chemical combination, giving rise to a substance that would be sufficiently stable to isolate as such. It is evident, however, in the present instances, that the combination is readily reversible. It probably represents a mutual adsorption, in which the two substances are held together by hydrogen bonds. The following observations concerning solubilities provide direct evidence, apart from a biological system, that sulfanilamide combines with urethane and certain other substances, in solution.

At 25°C., roughly 250 mg. per 100 cc. sulfanilamide crystallize out from a 1 per cent solution in distilled water or in phosphate buffered NaCl

solution. The crystals redissolve following the addition of urethane amounting to approximately 0.67 *M*. Moreover, although sulfanilamide is insoluble in ethyl ether, the presence of ether increases the water solubility of sulfanilamide, indicating the formation of at least a weak compound or complex between these three substances. Thus, the 250 mg. sulfanilamide which had been crystallized out from 100 cc. at 25°C., will go back into solution if it is then saturated with ether. The crystals will redissolve also following the addition of enough substance to make it up to 2 mol. acetone, 2 mol. glucose, 3 mol. urea, 4 mol. alcohol, or 20% peptone. The concentration of some of these is high, and thus the solvent may be considered as greatly altered, but the effect of combination on solubility is fundamentally the same. The combination possibly occurs by the formation of hydrogen bonds between the amino group of the sulfanilamide, and a carboxyl, ethoxy, or functionally equivalent group, of the other substances.

It may be remarked that the combination of sulfanilamide, and its derivatives, with various excretory products is undoubtedly responsible, in some measure, for maintaining the solubility of the drugs under conditions of unusual concentration *in vivo*, e.g., in the tubules of the kidney.

The type of combination between sulfanilamide and another substance which changes its solubility may or may not be identical with that which is chiefly responsible for decreasing its inhibitory action on an enzyme.

Diverse Sulfonamide Antagonists

A large number of substances have been found which, under appropriate conditions, will to a greater or lesser extent antagonize the sulfanilamide inhibition of bacterial growth. The most potent of these is *p*-aminobenzoic acid (Woods, 1940) which is generally believed to represent a constituent, essential to normal growth, with which sulfanilamide specifically competes, thereby exerting its inhibitory action. Other antagonists, which can hardly be considered as acting in such manner, include urethane and narcotics not derived from *p*-aminobenzoic acid. Still other antagonists include peptone (Lockwood, 1938), extracts of various tissues (MacLeod, 1940), coenzymes (West and Coburn, 1940), methionine (Bliss and Long, 1941; Harris and Kohn, 1941), amino acids other than methionine, and purines (Kohn and Harris, 1942; Martin and Fischer, 1942). Under proper conditions and con-

centrations certain of these same substances may "potentiate" the action of sulfanilamide, i.e., although exerting no apparent action alone, they increase the inhibition of the sulfanilamide. Others may be synergistic, i.e., exert inhibitions by themselves which may increase the sulfanilamide effect when mixed with it.

Obviously, various mechanisms must be involved in the antagonism, potentiation, and synergism which takes place among the diverse substances, acting on growth. Where the substance shows both an antagonistic and synergistic action, depending on concentration, and at the same time does not take part directly in a normal metabolic reaction, the mechanism concerned is quite possibly the same as that described above for mixtures of sulfanilamide and urethane, ether, et cetera. The theoretical derivations and formulations given in this paper provide a means of analysis, when applied to accurate data regarding the rate of the process in question, and when the site of action of the two substances is the same molecule.

When the antagonist of sulfanilamide takes part directly in a metabolic reaction, as, for example, by supplying added nutritive substrate or by acting as an intermediary in connection with some system of hydrogen transfer, the mechanism of antagonism is readily understandable on the basis of a stimulation in rate of the normal process. Such a mechanism may be responsible for the phenomenon observed, especially in an integrative process like growth, *even though the per cent inhibition due to sulfanilamide is greatly increased.* The sensitivity of luminescence to sulfanilamide offers a ready means of testing the effect of sulfanilamide in the presence of other substances. The action of the other substance alone may be critically examined by using washed cells, which readily indicate a nutritive or other influence in the normal metabolism. The experiments described below show that sulfanilamide combines with diverse substances, in both categories, namely, those which do and which do not take part directly in metabolism.

Sulfanilamide plus substances inert in metabolism. The ability of sulfanilamide to combine with or absorb upon substances which are insoluble, such as charcoal, powdered sulfur, etc., was tested by the effects of the mixtures on the luminescence of the psychrophilic species, *P. phosphoreum.* The presence of insoluble particles in suspension reduced the observed intensity of luminescence, without necessarily exerting a physiological reduction in the intrinsic brightness of each

cell. The former effect could be taken into account by the appropriate control without sulfanilamide. A more convenient procedure was simply to add a given weight, e.g., 100 mg. of the adsorbent or other insoluble agent, to a known volume of 0.006 M sulfanilamide in phosphate buffered NaCl solution. The mixture was agitated vigorously in a shaking machine at room temperature, then filtered. Five cc. of the filtrate was added to five cc. of the bacteria, and the intensity compared with a control tube without sulfanilamide. The validity of this method was checked by chemical analysis of the sulfanilamide content before and after exposure to charcoal.

TABLE III

Ability of Various Adsorbents and Other Substances to Remove Sulfanilamide from Solution, According to Inhibitory Action of Filtrate on Luminescence

Exp. 1 Temp. 12°C.	Control without sulfanilamide	Adsorbent 0	$BaCO_3$	Talc	Blood fibrin	Powdered sulfur	Permutit	Kaolin	Jack bean meal	Kieselguhr
Luminescence intensity	21.2	5.9	5.8	6.3	6.3	6.6	6.5	6.2	7.3	6.3
Per cent	100	27.8	27.3	29.7	29.7	31.0	30.6	29.2	34.4	29.7

Exp. 2 12.3°C.	Control	Adsorbent 0	"Darco"	Bone charcoal	Wood charcoal	CoO	ZnO	Lycopodium	Absorbent cotton	Blood fibrin
Luminescence intensity	15.1	4.9	7.6	10.2	4.8	4.3	5.4	5.3	5.0	5.2
Per cent	100	32.4	50.3	67.6	31.8	28.4	35.7	35.1	33	34.4

Exp. 3 12.5°C.	Control	Adsorbent 0	"Darco"	Bone charcoal	Wood charcoal	MgO	Al_2O_3	$BaSO_4$	Casein	Gelatin	Pumice	Soluble starch
Luminescence intensity	17.3	4.5	6.4	7.3	4.6	5.2	4.8	4.8	5.5	4.9	4.7	4.9
Per cent	100	26	37.0	42.2	26.6	30.0	27.7	27.7	31.8	28.3	27.0	28.3

The results summarized in Table III show that sulfanilamide may be adsorbed on diverse insoluble substances. Bone charcoal removes considerable quantities, Darco relatively much less, and wood charcoal practically none. Slight adsorption occurs on Jack bean meal and casein, and possibly, to an even less extent, on talc, powdered sulfur, lycopodium, permutit, kaolin, kieselguhr, (dried) blood fibrin, zinc oxide, and magnesium oxide. On the other hand, gelatin, soluble starch, pumice, barium sulfate and aluminum oxide apparently do not adsorb the drug to a measurable extent. Evidently, either the specific surface structure or chemical configuration may be the critical factor

determining whether combination of the sulfanilamide will occur. The diversity of substances above make it difficult to state any rule in this connection, but the fact that adsorption does take place on diverse materials is of general interest as well as possible significance in connection with physiological activity.

Nutrient substrates, amino acids, purines, etc. The luminescence in a suspension of washed cells is dim, and decreases logarithmically with time, unless a utilizable substrate is added. The addition of a suitable substrate causes the luminescence first to increase, then after a period of time that varies with species, temperature, et cetera, to decrease (Johnson, 1938, 1939). Thus, while some substrates can be metabolized much more readily than others (Johnson, 1936; Johnson, van Schouwenburg, and van der Burg, 1939) an increase in luminescence intensity following the addition of a given substance under conditions of substrate deficiency, indicates that the substance can be metabolized. Washed cells were used entirely in the experiments described below. The suspensions were placed in a water bath and measurements of luminescence were carried out at a relatively low temperature, favorable for revealing an antagonism of the type shown by urethane, ether, alcohol, etc., for sulfanilamide. At the same time, the temperature was not so low as to make the effect of adding nutritive substrates inconspicuous. The procedure used was simply to add equal portions of a given suspension of washed cells to a series of tubes in a water bath at a temperature between 12 and 14°C. The tubes contained an appropriate volume of buffered NaCl solution, sulfanilamide, other substance, and sulfanilamide plus other substance respectively, the solvent in all cases being buffered NaCl. Readings of luminescence were taken over a period of one hour, during which the temperature was constant. The luminescence intensity, however, was in some cases much less, e.g., without added substrate, and in other cases much greater, e.g., with added glucose, after this interval of time. The averaged readings are taken as an indication of the net effect and comparisons made on this basis.

The results are summarized in Tables IV to VIII. The concentration of sulfanilamide amounting to 0.003 M was most satisfactory for moderate inhibitions, and the other substances were tested on an equi-molecular basis. A complete analysis, of course, would necessitate an extensive study over an adequate range of both temperature and concentration, but the present data are sufficient to bring out some interesting points. Different concentrations and temperatures were studied only in a few

instances. The effects of added substances have been calculated as per cent in two ways: first, with respect to the primary control, consisting of washed cells alone, without addition of anything other than the salt solution. In this way, substances which increase luminescence above

TABLE IV

The Sulfanilamide Inhibition of Luminescence in Washed Cells of Bacteria, With and Without Added Amino Acids

Temperature = 12.7°C. Sulfanilamide 0.003 M; amino acids 0.003 M

Amino acid	0		Glycocoll		Arginine		Leucine		*dl*-Phenylalanine		Histidine	
Sulfanilamide	Control	+	0	+	0	+	0	+	0	+	0	+
Average luminescence over period of one hour	8.5	4.4	10.4	4.7	10.4	5.2	8.8	4.4	7.0	4.4	7.5	4.3
Per cent with respect to control	100	52	122	55.3	122	61	103.5	52	82.4	52	88	50.6
Per cent inhibition due to sulfanilamide	0	48	0	55	0	50	0	50	0	37.1	0	42.6

TABLE V

The Effect of Some Purines, Pyrimidines, and Creatinine on the Sulfanilamide Inhibition of Luminescence in Washed Cells

Sulfanilamide and added substances each 0.003 M in concentration. Temperature 14.0°C.

Substance added	0		Guanine		Hypoxanthine		Cytosine		Thymine		Uracil		Creatinine	
Sulanilamide	Control	+	0	+	0	+	0	+	0	+	0	+	0	+
Average luminescence, 1 hour	7.9	3.24	8.1	3.35	14.4	6.4	7.4	3.2	8.4	3.4	6.8	2.9	6.7	3.6
Per cent with respect to control	100	43.7	102	42.3	182	81	93.4	40.4	106	43	86	37	85	46
Per cent inhibition due to sulfanilamide	0	56.3	0	59	0	64	0	57	0	60	0	57	0	46

that of the control, even in the presence of sulfanilamide, are at once apparent. Secondly, the per cent inhibition resulting from sulfanilamide addition under a specific set of conditions is also given.

In general it will be noted that while glucose, peptone, and to a less

extent blood serum, over a wide range of concentrations, greatly increase the intensity of luminescence, other substances have relatively little

TABLE VI

The Effect of Glucose on the Sulfanilamide Inhibition of Luminescence in Washed Cells

Sulfanilamide 0.003 *M*. Temperature 12.5°C.

Glucose concentration	0		0.02 *M*		0.01 *M*		0.005 *M*		0 0025 *M*		0.00125 *M*	
Sulfanilamide	Control	+	0	+	0	+	0	+	0	+	0	+
Average luminescence for 1 hour	1.09	0.66	11.7	4.1	12.3	4.3	10.5	3.7	9.4	3.9	12.9	4.8
Per cent with respect to control	100	60.8	1,075.0	376	1,130.0	394.0	964.0	339.0	862.0	358.0	118.0	440.0
Per cent inhibition due to sulfanilamide	0	39.2	0	65	0	65	0	64.8	0	58.5	0	62.7

TABLE VII

The Effect of Peptone on the Sulfanilamide Inhibition of Luminescence in Washed Cells

Sulfanilamide 0.003 *M*. Temperature 13.8°C.

Conc. Peptone	0		0.8%		0.4%		0.2%		0.1%		0.05%	
Sulfanilamide	Control	+	0	+	0	+	0	+	0	+	0	+
Average luminescence for 1 hour	3.0	1.94	14.9	4.1	16.4	4.5	15.8	5.1	12.3	4.8	9.6	4.6
Per cent with respect to control	100	64.7	497.0	137.0	547.0	150.0	527.0	170.0	411.0	160.0	320.0	153.0
Per cent inhibition due to sulfanilamide	0	35.3	0	72.5	0	72.5	0	67.7	0	61	0	52.2

TABLE VIII

The Effect of Human Blood Serum on the Sulfanilamide Inhibition of Luminescence in Washed Cells

Sulfanilamide 0.003 *M*. Temperature 14°C.

Conc. Blood serum	0		20%		10%		5%		2.5%		1.25%	
Sulfanilamide	Control	+	0	+	0	+	0	+	0	+	0	+
Average luminescence for 1 hour	5.2	2.5	17.8	5.6	16.8	4.8	14.3	4.6	10.8	4.7	8.3	4.0
Per cent with respect to control	100	48	285.0	108.0	323.0	92	275.0	88.5	208.0	90.4	160.0	77
Per cent inhibition due to sulfanilamide	0	52	0	62	0	71.5	0	68	0	66.5	0	51.7

effect.. Glycocoll and arginine increase luminescence to a slight extent, and hypoxanthine much more. Phenyl alanine, histidine, uracil, and

creatinine cause slight inhibitions for reasons not entirely clear, although like certain glucosides (Johnson, 1937) they possibly exert a competitive inhibition against normal metabolites. A number of experiments were carried out with different preparations of methionine, but the results have been inconclusive, and no very pronounced effect of this substance on luminescence, with or without sulfanilamide, has been observed.

In most cases a pronounced increase in luminescent metabolism results in an increase in the per cent inhibition due to sulfanilamide. In other words, the sulfanilamide inhibition is generally less when the metabolism is low. Yet the absolute rate of metabolism, in the presence of a suitable substrate plus sulfanilamide may be much greater than that of similar cells, without both substrate and sulfanilamide. Thus, the effect on an integrative process, such as growth, might in the course of time amount to a strong antagonism of the sulfanilamide inhibition.

Discussion

The foregoing results are of interest first of all in providing a quantitative, theoretical basis for the antagonism and synergism of diverse inhibitors acting upon the same system. This theory has been made possible through the new understanding of the action of the separate inhibitors, particularly urethane and sulfanilamide, in relation to concentration, temperature, and hydrostatic pressure (Johnson, Brown, and Marsland, 1942; Johnson, Eyring, and Williams, 1942; Johnson and Eyring, 1943). From an understanding of the action of the two separately, and a knowledge of their tendency to combine or not to combine with each other, it is possible to predict with considerable accuracy the effects of the two substances when simultaneously present. It is also possible to predict the effects of temperature on their combined effect, and to account for the fact that the same substance may show an antagonistic action against a given inhibitor at one temperature, and a synergistic action at a different temperature. The general implications of these facts with their theoretical basis, in relation to the action of diverse drugs, poisons, and indifferent inhibitors, are obvious, but it should be emphasized that the theory outlined deals entirely with the particular situation in which the several inhibitors all act on the same molecule. It is to be expected that in many cases, more than one system will be affected by a given inhibitor, thus making the situation more complicated than that dealt with above. Moreover, it is possible, though on *a priori* considerations not likely, that a given inhibitor, such as sulfanilamide,

which acts in a manner conforming to Type I, in one catalytic system, may act in a manner resembling Type II in another system, or series of systems concerned in a complex phenomenon, such as growth. Adequate data for analysis of the action of these inhibitors on other, well defined, systems remain to be provided.

The results described above, in relation to luminescence, make it clear that there are several distinct ways in which the sulfanilamide inhibition of a metabolic reaction may be antagonized. There is reason to believe that the same considerations apply to growth. Thus, the following general categories may be recognized on the basis of the mode of antagonism:

(1) *Antagonism of sulfanilamide inhibition by an inhibitor of a different type, e.g., urethane, which combines with the sulfanilamide in the manner discussed above.* Some antagonism should become apparent even if the two inhibitors do not act on the same molecule. The elucidation of the urethane antagonism of the sulfanilamide inhibition of luminescence is provided for the first time herewith, and application of the same theory to other cases remains to be tested. It seems likely, however, since sulfanilamide enters into a loose compound formation with urethane, acetone, ether, etc., and may be adsorbed to some extent by a variety of inert substances such as bone charcoal, permutit, etc., that an antagonism of sulfanilamide inhibitions by bacterial and tissue products may sometimes occur in a similar manner. On the other hand, the mechanism involved may be according to one of the other more or less generally recognized modes of antagonism listed below.

(2) *Antagonism of sulfanilamide inhibition by substances which assume the rôle of a catalyst or act in conjunction with a catalyst.* Possibly the effects of coenzymes (West and Coburn, 1940) and of *p*-aminobenzoic acid (Woods, 1940) may be accounted for on this basis, although the specific reaction or group of reactions involved remain to be identified. Metabolic and tissue products might be expected frequently to contain small amounts of oxidizable-reducible compounds or other substances which might reduce the effectiveness of sulfanilamide by means of facilitating a normal catalytic reaction.

(3) *Antagonism of sulfanilamide inhibition by substances which may be metabolized in the manner of a normal substrate, such as glucose.* Possibly in this category may be included small amounts of peptone (Lockwood, 1938) under certain conditions, and also some of the amino acids. Under appropriate conditions, it should be possible to demonstrate that many

individual substances which may act as nutritive or oxidizable substrates are capable of antagonizing the inhibition of growth by sulfanilamide.

Under the complex conditions accompanying infections, it might be expected that substances in all of the above categories have an influence on the action of sulfanilamide in stopping or slowing the growth of bacteria. For purposes of analysis, it should be useful to recognize, if possible, which of the three general categories a given antagonist represents. If it belongs to the first group, the theory and formulations presented in this paper provide a basis for reaching a satisfactory analysis, and for making certain significant predictions.

Summary

On the basis of theoretical considerations, formulations have been derived for the effects of inhibitors which combine reversibly with an enzyme to give two products, as with the active and reversibly heat inactivated forms, respectively (Type I), and for inhibitors which combine similarly with the enzyme, but in a manner that gives a single product (Type II). The equilibria that result when mixtures of the same type are present, as well as mixtures of different types, are considered at length, and formulations are derived for testing the conformity of the data to the particular case involved.

When added alone, sulfanilamide conforms to Type I and urethane to Type II in bacterial luminescence, with each evidently acting directly on the light-emitting oxidative system. When simultaneously present, the sulfanilamide combines with the urethane, which is present in excess. As a result, at low temperatures, urethane is strongly antagonistic to the sulfanilamide inhibition, whereas at higher temperatures it is synergistic, because the urethane inhibition itself increases with temperature.

With the aid of the theoretical formulations, and data concerning the effect of sulfanilamide and urethane on luminescence, separately and in mixtures, an equilibrium constant for the reversible combination, or mutual adsorption of urethane and sulfanilamide, may be calculated. Using this constant, which is approximately 50, it is possible to predict with considerable accuracy the quantitative effect of various concentrations of the two inhibitors when mixed in all proportions.

Other inhibitors, presumably of Type II, including ethyl alcohol, butyl alcohol, chloroform, ether, and acetone, also give evidence of forming a combination or complex with sulfanilamide in solution, with the result that they tend to exhibit in low concentrations an antagonistic action at

low temperatures, and synergistic action at higher temperatures. In all cases, the amount of inhibition depends both on concentration and on temperature.

The solubility of sulfanilamide is increased by urethane, alcohol, chloroform, acetone, glucose, peptone, and ether. In ether alone sulfanilamide is largely insoluble.

Adsorption of sulfanilamide takes place on diverse substances according to the specific character of the surface or chemical constitution. Using bacterial luminescence as an indicator of concentration, it is apparent that bone charcoal takes up considerable amounts of sulfanilamide from neutral solutions, "Darco" adsorbs less, and wood charcoal practically none. Slight amounts appear to be taken up by Jack Bean meal, casein, and very slight amounts by talc, powdered sulfur, lycopodium, kaolin, kieselguhr, dried blood fibrin, zinc oxide, and magnesium oxide, while no amount detectable with luminescence is adsorbed by gelatin, soluble starch, pumice, barium sulfate, or aluminum oxide.

Using washed cells it was shown that various substances which can be metabolized by the bacteria influence the per cent inhibition of luminescence in the presence of a given concentration of sulfanilamide. In general, substances giving rise to a considerable increase in luminescent metabolism, cause the per cent inhibition due to sulfanilamide to increase, e.g., glucose, peptone, blood serum, hypoxanthine, glycocoll, and arginine. The absolute intensity, however, of luminescence in the presence of these substances plus sulfanilamide, was greater than that of corresponding portions of the washed cell suspension with neither sulfanilamide nor added substrate. Under the experimental conditions, substances which had very little or doubtful effects, either on the luminescence or the sulfanilamide inhibition of luminescence in washed cells, include leucine, guanine, and thymine, while slight inhibitions, for reasons not clear, resulted from phenyl alanine, histidine, uracil, and creatinine.

The results are discussed at length in regard to the general significance of antagonism and synergism among diverse inhibitors, as well as the distinct modes of antagonism of sulfonamide inhibitions of metabolic reactions.

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Fructose-1,6-Diphosphoric acid and Fructose-6-Monophosphoric Acid

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Introduction

The pure, solid salts of fructose-1,6-diphosphoric acid with inorganic bases which have been isolated are derivatives of the tetrabasic acid $C_6H_{10}O_4(PO_4H_2)_2$. Its alkaline earth salts are difficultly soluble in water with the exception of the magnesium salt, $C_6H_{10}O_4(PO_4Mg)_2$, which is readily soluble in water, well characterized and very stable (1). The alkali metal salts have been described as sticky masses which decompose on standing (2). Neither these compounds nor the insoluble zinc or calcium salts are definitely crystalline (3). There is no doubt, however, that stable acid salts exist in which fructose-diphosphoric acid acts as a di- and tribasic acid. These are the strychnine salt, $C_6H_{10}O_4(PO_4H_2)_2 \cdot (C_{21}H_{22}N_2O_2)_2$, formerly described by Neuberg and Dalmer (4), and the benzidine salt, $C_6H_{10}O_4(PO_4H_2)_2 \cdot (H_2N \cdot C_6H_4 \cdot C_6H_4 \cdot NH_2)_3$, isolated by Neuberg and Scheuer at an earlier date (3). Both of these salts crystallize very well.

When the normal barium or calcium salts of hexose-diphosphoric acid, which are only slightly soluble in water, are purified by the method of Neuberg and Sabetay (1)—solution in acid and reprecipitation by neutralization—it has been observed that they are dissolved long before four equivalents of acid have been used. This observation and the isolation of the acid salts mentioned above indicate the existence of soluble acid alkaline earth salts.**

* Abridged from a thesis submitted by Mortimer A. Rothenberg in partial fulfillment of the requirements for the degree of Master of Science, New York University, 1942.

** Their existence and use have been outlined briefly by Neuberg and Kobel in their "Methoden zur Untersuchung von Gärflüssigkeiten, Handbuch der Pflanzenanalyse, vol. IV, p. 1279, Springer, Vienna (1933). Later on the late Professor Robison seems to have observed an occurence of acid salts also. We found a notice, but no description in a paper by Herbert, Gordon, Subrahmanyan, and Green. [*Biochem. J.* **34**, 1109 (1940)].

Details on the preparation of the acid Barium salt $C_6H_{10}O_4(PO_4Ba)$ (PO_4H_2), resp. $C_6H_{10}O_4(PO_4H)_2Ba$, in pure solid form will be given in the Experimental part; and these directions must be closely followed. When kept dry, this salt was found to be stable for months. Being easily water-soluble, it is suitable for double decompositions. We likewise have reason to believe that there are tribasic alkaline earth salts.

Pure starting materials must be used to obtain an absolutely pure compound. We used a pure tetrabasic barium fructose-1,6-diphosphate obtained from the crystalline strychnine salt. The pure neutral and acid barium salts were used to redetermine optical activity, reducing action with Ost solution, and behaviour against bromine water.

To increase accuracy we worked with high concentration and found the following for the free acid: $[\alpha]_D^{17} = +4.04°$ to $+4.15°$, as compared with the former figures (5) $[\alpha]_D^{15} = +3.55°$.

Power of reduction: 48% of pure fructose.

Although comparison with fructose appears to be most logical, the majority of the comparisons have been made with glucose. In addition, reductions were also carried out in the strongly alkaline medium of Fehling's or Bertrand's solutions. We used the bicarbonate-carbonate solution of Ost. There are definitely less secondary alkali effects in Ost solution. Furthermore, the sensitivity is higher, as the sugars precipitate almost twice the quantities of Cu_2O from Ost solution as compared with alkaline mixtures of copper tartrates. The unused portion of copper salt can be determined conveniently iodometrically according to the method of de Haen-Lehmann-Maquenne. In this way the quantity of Cu_2O produced is determined, and the equivalent of sugar is taken from the tables of Ost (6). We recommend this exact, sensitive, and simple method for similar purposes. Ost solution keeps almost indefinitely. Furthermore, this method is superior to most others, as substances such as simple carbonyl compounds, ureids of sugars, and various other materials which react with Fehling's solution are inert with Ost solution. Inorganic phosphates formed by boiling Ost solution with esters of sugar-phosphoric acids will remain in solution.

Though the properties of the salts prepared by the ordinary method after thorough purification seem to indicate that there is but one fructose-diphosphoric acid, Meyerhof and Lohmann (7) assumed that there was a mixture with an aldose compound, basing their supposition on the use of iodine in NaOH. This seems understandable, as these authors were using the original method of Willstätter and Schudel. Auerbach and Bodlaender (8) substituted a suitable $NaHCO_3$-Na_2CO_3 buffer for the NaOH, whereas Macleod and Robison (9) used Na_2CO_3. In a bicarbonate solution fructose-diphosphate requires practically no more iodine than pure fructose. Robison (9), therefore, rightly doubted

whether this compound contained any aldose derivative. A partial Lobry de Bruyn transformation may be effected by treating a ketose in a carbonate alkaline solution, particularly, if the keto-enol equilibrium is constantly shifted by oxidation with hypoiodite of the aldose formed. The Willstätter-Schudel method has been frequently criticized as to conclusiveness in regard to ketoses.

Fructose may require little hypoiodite under favorable conditions, but this is not general, as has been shown, for example, by Neuberg and Collatz (10) in the case of dihydroxyacetone, by Pasternak (11) for inosose, and by Schmidt and Heintz (12) for *d*-xylo-ketose. These ketoses partly utilize iodine even in bicarbonate alkaline solution at a rate which would seemingly indicate the presence of 25 to 100% of aldose.

These drawbacks have been excluded in the method of Neuberg and Collatz (10), by oxidizing with bromine water in an acid medium. Even an extremely sensitive ketose, such as dihydroxyacetone, is not affected, whereas the isomeric aldose, glyceraldehyde, is completely oxidized. Meyerhof and Lohmann (13) subsequently applied this method, in which transformations due to hydroxyl ions are excluded, in determining triose derivatives.

If pure fructose-diphosphate is subjected to the bromine water treatment, no noticeable oxidation occurs. The presence of an aldose compound can, therefore, be excluded. (Glucose-6-phosphoric ester is completely oxidized to 6-phospho-gluconic acid by bromine water (14)).

In 1918, Neuberg (15) obtained fructose-6-phosphate by partial hydrolysis of fructose-1,6-diphosphate. The rôle of this compound in the synthesis, degradation, and transformation of sugars was discovered when this fructose-monophosphate was also found present in nature (16), *i.e.*, in carbohydrate transformations by microorganisms, by animal tissues, and by green leaves. This compound is still prepared by the method described in 1918. The calcium or barium salt of fructose-1,6-diphosphate is boiled for 30 to 60 minutes with dilute HCl, H_2SO_4, or oxalic acid. The phosphoric group in position 1 is split off more readily than that in position 6 by partial hydrolysis. The temperature used, 100°C., causes of course, destruction of some of the fructose monophosphate, and the resulting free ketose is decomposed to produce a yellow tint. This disadvantage can be overcome by carrying out the partial hydrolysis of calcium fructose-1,6-diphosphate with *N*-HCl (or, in the case of the barium salt, with *N*-HBr) at 35°C. After about 5 days, approximately half the weight of the original diphosphate is ob-

tained as the alkaline earth salt of the monophosphate. If the starting material was pure the fructose-6-phosphate is equally pure. The compound, when absolutely pure, does not require any bromine, showing that no isomerization into an aldose derivative is taking place, as was assumed earlier (17).

Its *optical activity* has been determined in the form of its pure barium salt:

$[\alpha]_D^{19} = +3.58°$, as compared with the former measurement $[\alpha]_D^{17} = +2.9°$.

Experimental

Pure d-fructose-1,6-diphosphate

The starting material was neutral barium-hexose-diphosphate obtained according to earlier data (18). The acid strychnine salt (4) was prepared next. 52.2 g. of the quadruply recrystallized alkaloid salt were dissolved in 20 times their weight of 90 per cent methanol. After this solution had cooled, but prior to the crystallization of the strychnine salt, a solution of $Ba(OH)_2 \cdot H_2O$ in methanol* was added with vigorous shaking.

We used 199 cc. of a clear filtered solution containing 94.75 g. $Ba(OH)_2 \cdot H_2O$ in 1000 cc. of water-free methanol. This represents 99.5 per cent of the quantity required for the transformation of the tetrabasic barium salt. This is quantitatively precipitated on addition of 100 cc. of acetone, but contains free strychnine. The sedimented precipitate is separated by centrifugation, then extracted with vigorous shaking first with a mixture of 1 part of acetone and 9 parts of methanol, then with ethanol, and finally with chloroform containing 5 per cent of methanol, until the strychnine tests of Mandelin and Sonnenschein are negative. The barium hexose-diphosphate is absolutely pure. It appears as a fluffy, snow-white powder. It is definitely soluble in ice water, easily soluble in solutions of neutral ammonium salts at room temperature (1), and does not contain any trace of inorganic phosphate or nitrogen containing impurities. Small quantities, dried in a high vacuum

* Instead of the solution formerly used by Neuberg and coworkers [*Biochem. Z.* **1**, 166 (1906); **9**, 537 (1908); **12**, 337 (1909); **170**, 258 (1926)] containing BaO or $Ba(OH)_2 \cdot 8H_2O$ in methanol, the commercial monohydrate can be used advantageously. It is cheaper than BaO and its methyl alcoholic solution contains less water than in the case of the octohydrate.

at 30°C. over $CaCl_2$ and paraffin, were found to consist of the water-free salt: $C_6H_{10}O_4(PO_4Ba)_2$. It is difficult to obtain larger quantities of the water-free substance. Drying in ordinary vacuum at room temperature over $CaCl_2$ and paraffin produces a salt containing mostly 1 mole of H_2O.

Optical Activity. 6.108 g. of water-free barium salt were dissolved in 20 cc. of 2*N* HBr and made up to 25 cc. with water, corresponding to 3.40 g. of free fructose-diphosphoric acid: $[\alpha]_D^{17} = +4.04°$ ($\alpha = +1.10°$, $l = 2$, $c = 13.6$).

After standing for 2 hours in a refrigerator at 3°C., these values remained practically unchanged.

Reducing Power: see page 38.

Reaction with Bromine Water. 0.3055 g. of the salt were dissolved in 10 cc. *N*/10 HBr as calculated for the acid salt. 200 cc. *M*/100 bromine water containing 10.0 g. of KBr were then added. After this mixture was kept in the dark in a tightly glass-stoppered bottle for 7 days at room temperature, no measurable quantities of bromine had been used. The following is to be concluded from these experiments: Though part of the ester is hydrolyzed during the experiment, neither fructose phosphate nor free fructose use up bromine under these conditions.* Glucose, however, reacts rapidly.

Acid Barium Salt of d-fructose-1,6-diphosphoric Acid. To 6.29 g. of the tetrabasic barium salt, 50 g. of chopped ice and 50 cc. of 0.4 *N* HBr solution at 3°C. were added. The resulting clear solution is immediately added dropwise, with constant stirring, to 600 cc. of ethanol. After keeping for 5 minutes at 3°C., the precipitate is filtered by suction and washed with alcohol, to eliminate the $BaBr_2$, which is readily soluble in alcohol. The salt is then washed with alcohol and ether, slightly dried *in vacuo*, dissolved in 70 cc. of cold water, reprecipitated with 400 cc. of cold alcohol, quickly filtered by suction, washed with pure ethanol and finally with water-free acetone. The alcoholic filtrate does not contain any free phosphoric acid if the temperature has been kept down to 3°C.

* Instead of the solution of bromine in KBr, acidified bromide-bromate mixtures may be used in analogy to the old method of titration of phenols by W. F. Koppeschaar [*Z. anal. Chem.* **15**, 233 (1876)]. If a mixture of $CaBr_2$ and $Ca(BrO_3)_2$, or the corresponding strontium or barium salts are used, this procedure may be successfully applied preparatively in the carbohydrate group. This will be the subject of a later paper.

However, the solution contained nearly exactly 1 mole of $BaBr_2$. (If the acid salt is prepared at a higher temperature, a small amount of inorganic phosphoric acid will be found in the alcohol solution.) The substance was twice reprecipitated by acetone from an aqueous solution, and washed with acetone and then with ether. The substance, dried in a high vacuum over P_2O_5 and paraffin, remains snow-white and has the formula: $C_6H_{10}O_4(PO_4H)_2Ba$.

$C_6H_{12}O_{12}P_2Ba$ (475.5):
Calculated: Ba = 28.9, P = 13.0, C = 15.1, H = 2.5.
Found: Ba = 29.1, P = 12.9, C = 14.65, H = 3.0.

The substance is very soluble in water (about 1:2), the solution having an acid reaction.

The solution of the acid salt reacts as follows: With $Ba(OH)_2$ the sparingly soluble tetrabasic salt is formed. Precipitate formed with NH_4OH, soluble in ammonium salts. Precipitate with normal and basic lead acetate, scarcely soluble in an excess of reagents. With cupric acetate blue-white precipitate, soluble in KOH or $KHCO_3$ with deep blue color; the solution gives the Trommer test. Crystalline precipitate with benzidine hydrochloride and on boiling with phenylhydrazine acetate (the osazone derivative). No precipitate with NO_3Hg, $(CH_3COO)_2Hg$, $AlCl_3$, $FeCl_2$. By careful addition of $Fe(NO_3)_3$, as neutral as possible, a yellowish white precipitate, soluble in KOH or K_2CO_3 with reddish yellow-color. No precipitate with $AgNO_3$, but thick white sediment after subsequent neutralization with NaOH.

Optical Activity. 10 cc. of 2*N* HBr were added to 4.755 g. of the water-free barium salt and made up to 25 cc. with water. This solution contained 3.4 g. of free *d*-fructose-1,6-diphosphoric acid. $[\alpha]_D^{18} = +4.15°$ ($\alpha = +1.13°$, l = 2, c = 13.6).

The free fructose-diphosphoric acid shows practically the same polarimetric values as those obtained on starting from the tetrabasic salt.

The acid salt shows the same resistance to *bromine water* as the neutral substance. There is practically no absorption of bromine.

The *reducing power* was determined as follows: 0.4755 g. of substance were transformed in aqueous solution with the exact amount of K_2SO_4. The solution was brought to 75 cc., and 25 cc. of the clear filtrate were used for the determination according to Ost (50 cc. copper solution, time of boiling 10 minutes; comparison with a solution containing 0.180

g. of pure *d*-fructose* in 75 cc. of water). Reducing power 48 per cent compared with 100 per cent for pure fructose.**

The cold aqueous solution of the acid barium salt is rather stable. When brought to a boil, free sugar and inorganic phosphate are formed after a short time. If the dry substance is heated in water-free methanol, a reducing compound is found in the solution, but there are neither barium- nor phosphate ions. The acid reaction shown by the methanol solution is due to a percentage of free fructose-diphosphoric acid. If the clear liquid is neutralized with methyl alcoholic $Ba(OH)_2$, using phenolphthalein as indicator the slightly soluble tetrabasic salt is precipitated. This was identified by its solubility in ice water and in neutral ammonium salts, by its power of reduction, by the presence of organic phosphoric acid only, and by the Seliwanoff test. The substance is insoluble in warm methanol, does not contain any inorganic phosphate, is easily water-soluble, and therefore not the tetrabasic salt. This may be explained in the light of a disproportion according to the following formulation:

$$3\,C_6H_{10}O_4(PO_4H)_2Ba = C_6H_{10}O_4(PO_4H_2)_2 + (BaO_4P\cdot C_6H_{10}O_4\cdot PO_4H)_2Ba$$

Free fructose di-phosphoric acid and its tribasic salt are formed. This reaction may be compared to the transformation of the primary alkaline earth phosphates into secondary alkaline earth phosphates and free phosphoric acid, for instance:

$$(H_2PO_4)_2Ca = H_3PO_4 + HCaPO_4$$

This reaction shows that the *tribasic alkaline earth salts of fructose diphosphoric acid* are equally soluble in water. Just 1 mole of HBr will transform the slightly water-soluble $C_6H_{10}O_4(PO_4Ba)_2$ into an easily water-soluble salt, precipitable by ethanol, or methanol containing acetone. Its reactions are similar to those of the dibasic salt, but we could not obtain a satisfactory analysis for the tribasic salt.

If normal barium fructose-diphosphate which had not been purified

* Pure *d*-fructose, required for this comparison, is obtained by repeated recrystallization in water-free methanol according to Ost (6), or especially to H. O. L. Fischer and E. Baer [*Helv. Chim. Acta* **19**, 528 (1936)]. Addition of 5% methylacetate after complete solution of the sugar in warm methanol will hasten the crystallization.

** The result of *K. Freudenberg* [*Z. angew. Chem.* **42**, 295 (1929)] agrees with this value. He found 41–43% of the reducing power of glucose by the Fehling method. Other authors report lower values.

by transformation into the strychnine compound is used, the analytical value obtained are not good. This is also true of the *acid calcium salt*, which may be prepared in the same manner as the acid barium salt by HCl or HBr. The calcium salt is at first slightly hygroscopic, but can be obtained as powdery substance after repeated reprecipitation by alcohol from an aqueous solution. The analysis of this easily water-soluble compound was unsatisfactory, no matter whether the substance had been prepared by means of HBr or HCl. The substance did not contain any halogen, as $CaCl_2$ and $CaBr_2$ are very soluble in alcohol and did not form any double salts.

The fact that fructose-diphosphoric acid forms acid alkaline earth salts which are precipitated by methyl or ethyl alcohol, may be applied to the purification of raw fructose-diphosphate. If, for instance, the barium salt contains inorganic phosphate or is mixed with monoesters, it is dissolved at low temperature in as much HBr, $HClO_4$ or $CCl_3 \cdot CO_2H$ as is necessary to make sure that all acid equivalents not pertaining to the hexose-diphosphoric acid are set free, *i.e.*, practically in just enough acid to dissolve the entire amount of substance. Any acid is suitable, provided its alkaline earth salts are soluble in alcohol. The alkaline earth perchlorates are even soluble in acetone. If alcohol is then added for precipitation, H_3PO_4, $BaBr_2$ and impurities will remain in solution, including the free monophosphoric esters. If the corresponding strontium salt is used, HCl or $CH_3 \cdot CO_2H$ will do, as their strontium salts are sufficiently soluble in alcohol. The acid barium- or strontium fructose-diphosphate is then dissolved in water, reprecipitated by alcohol or acetone if necessary, filtered by suction and transformed in aqueous solution by $Ba(OH)_2$ or $Sr(OH)_2$ into the nearly insoluble tetrabasic salts which are obtained pure by this treatment (see Neuberg and Kobel, footnote page 32).

d-Fructose-6-phosphate

a) 13 g. of the calcium fructose-diphosphate are dissolved in 150 cc. of HCl. The solution is placed in an incubator at 35°C. After 5 days the solution is cooled and the acid neutralized with $CaCO_3$ to congo red. A fine suspension of $Ca(OH)_2$ is then added until a weakly alkaline reaction to phenolphthalein is obtained. Immediately thereafter, CO_2 bubbled through the solution. The mixture is warmed in a vigorously boiling water bath for 3 minutes and filtered by suction while still hot. The precipitate is washed with hot water. The filtrate is then concentrated *in vacuo* at 35°C. to about 90 cc. The concentrate is filtered and added slowly in a thin stream to four times its volume of 95 per cent alcohol with vigorous stirring. Flocculation of the precipitate may be accomplished by allowing it to stand in the refrigerator over night. After filtering by suction, the precipitate is washed with 95 per cent alcohol and dried in a vacuum desiccator. The substance is free of

chloride. It is dissolved in 70 cc. of water and dropped into 275 cc. of 95 per cent ethanol.

If a solution containing 5 per cent of the salt filtered by suction becomes opaque on boiling, it indicates presence of hexose-diphosphate. In this case, the entire amount of substance is dissolved in 150 cc. of water, and 10 cc. of absolute alcohol are added. By boiling on a water bath for a short time the diphosphate is precipitated and filtered off while hot. The solution is again concentrated *in vacuo* and treated as previously described. Yield: 6 to 6.5 g.

b) *Barium fructose-6-phosphate* is prepared analogously, hydrolysis being performed with N HBr. Owing to the solubility of $BaBr_2$ in alcohol, the substance is obtained free of halogen. The barium salt will sediment even more readily than the calcium salt.

Optical Activity. 2.010 g. of water-free barium salt are dissolved in water and brought to 20 cc. $[\alpha]_D^{19} = +3.58°$. ($\alpha = +0.72°$, $l = 2$, $c = 10.05$.)

This substance may contain a small amount of the isomeric fructose-1-phosphate. Though in fructose-1,6-diphosphoric acid, the phosphoric group in position 6 is removed much more slowly than that in position 1, there is still a possibility that a small amount may accumulate. The careful treatment at 35°C. seems to yield a purer fructose-6-phosphate. The higher dextro-rotation supports this assumption. The *d*-fructose-1-phosphoric acid is strongly levorotatory (19).

Reducing Power. The theoretical amount of K_2SO_4 was added to an aqueous solution of 0.3955 g. of substance which had been made up to 75 cc. The $BaSO_4$ was filtered off, and 25 cc. of the filtrate used for determination as indicated previously. The reducing power is 82 per cent of the equivalent of free *d*-fructose.

Reaction with Bromine Water. Neither the aqueous solution of the barium salt, nor its solution in the equivalent amount of 0.1 N HBr used more than traces of bromine after standing for 7 days at room temperature.

The resistance of fructose-6-phosphate to the action of bromine water is remarkable. (Part of the free ester is saponified after several days of digestion. Some free H_3PO_4 is then present, but by no means all of the phosphoric acid is mineralized.) It is known (20) that fructose does not resist oxidizing agents under the most varied conditions. It is even altered by the oxygen of the air, particularly in mixtures of phosphates in the presence of heavy metals capable of forming complexes, such as Fe and Mn (Spoehr, 21), and will evolve CO_2. Meyerhof and Lohmann (22) observed a high degree of oxidation of fructose-6-phos-

phate at pH 8 in the presence of traces of copper. They did not mention any products of oxidation other than CO_2. Neuberg and Collatz (23) carried out an almost quantitative degradation of fructose-6-phosphoric acid at room temperature by oxidation with molecular oxygen in $Ba(OH)_2$, and obtained *d*-arabonic acid-5-phosphate which is very resistant to acids and alkalies, but is completely hydrolyzed by animal or plant phosphatases. The conditions for the oxidation and splitting off the carbon atom in position 1 of fructose-6-phosphate apparently do not prevail in the acid medium of the bromine water. Bromine in alkaline solution (hypobromite) splits off a considerable amount of phosphoric acid. As mentioned above, Neuberg and Collatz (23) found molecular oxygen suitable for degradation of phosphorylated hexoses into the corresponding pentose derivatives, *i.e.*, shortening the carbon chain without splitting off the phosphate group. The *d*-arabonic acid-5-phosphoric ester obtained by this method is an epimer of the oxidation product of natural *d*-ribose-5-phosphate obtained by Levene and Jacobs (24), and may be contained in the mixture of substances resulting from *d*-phospho-gluconic acid subjected to enzyme systems of yeast by Dickens (25).

The simple manner of preparing *d*-fructose-1,6-diphosphoric acid according to Neuberg and Lustig (18) and the method for its purification as described in this paper, serve as a convenient method of preparing pure *d*-fructose-6-phosphoric acid. The latter is more easily accessible than the isomeric *d*-glucose-6-phosphate, the Robison ester. In many cases the so-called Neuberg ester is a suitable substrate not only for the study of phosphatases but for certain problems of carbohydrate metabolism as has been successfully proven by Kuhn and coworkers, Wagner-Jauregg (27), and other. Further applications will be found, since the mechanism of the mutual transformations of phosphorylated hexoses into one another has been clarified (16), and since Meyerhof as well as Cori and Cori have shown the reversibility of nearly all reactions affecting phosphorylated sugar derivatives.

Du Bois and Potter [*J. Biol. Chem.* **147**, 41 (1943)] proposed recently to prepare from a single fermenting mixture fructose-diphosphate, Robison ester, and phosphoglyceric acid, in a procedure as it has been applied for the analytical elucidation of the formation of phosphorylated products of sugar degradation. This modus procedendi is not commendable for preparative purposes. The optimal conditions for obtaining maximum yields are never the same for each of these esters, as it is known from earlier publications by *Harden, Kobel, Leibowitz, Lustig, and Neuberg*. A comparison is possible if the yields are calculated from the phosphate applied. Then it follows that the yields on little purified material

obtained by *Du Bois and Potter* stay considerably behind the yields on far more pure products obtained from separate batches.—The preparation of phosphoric esters from fresh yeast is described, for instance for hexose-diphosphate, in 1932 already, the preparation of pure phosphoglyceric acid with fresh yeast, without addition of hexosediphosphate was found in 1933. The procedure by *Ostern and Guthke* (1937) is identical with the one by *Neuberg and Kobel* (1933); the preliminary fermentation without phosphate, proposed by the Polish authors, may be of value in the case of a yeast with weakened fermenting power, but is useless if normal yeast is used. [Ref.: Harden, Alcoholic Fermentation p. 50 (1932); Kobel and Neuberg, Handbuch der Pflanzenanalyse, vol. II, p. 554 (1932); Neuberg u. Lustig, *Arch. Biochem.* **1**, 311 (1942).]

Summary

1. From the acid strychnine salt of *d*-fructose-1,6-diphosphate, the pure tetrabasic barium salt was prepared. This material served for determination of the constants. Rotatory power $[\alpha]_D^{17}$ +4.04° to +4.15° (for the free acid). Reducing power (alkali salt): 48 per cent of that of *d*-fructose. Absolute resistance against bromine water.

2. From the neutral barium salt $BaO_4P \cdot C_6H_{10}O_4 \cdot PO_4Ba$ the secondary acid barium salt $BaO_4P \cdot C_6H_{10}O_4 \cdot PO_4H_2$ was prepared. This is very soluble in water (about 1:2) in contradistinction to the neutral salt (1:170). It can be precipitated quantitatively by methanol, ethanol, and acetone. It is very stable, free from impurities and readily yields double transformations. Because of its solubility in water and precipitability by alcohols and acetone it is suitable for separation of fructose-diphosphate from inorganic phosphates and other by-products. It can be easily converted to the tetrabasic salt.

3. A simple preparation of *d*-fructose-6-monophosphate is given by the hydrolysis of the *d*-fructose-1,6-diphosphates with HCl or HBr at 35°C. under special conditions. The alkaline earth salts of this monophosphoric ester were also prepared in the pure state. They are free from contamination with isomeric aldose derivatives. The *d*-fructose-6-monophosphate is absolutely resistant to bromine water. $[\alpha]_D^{19}$ = +3.58° (Ba-salt); reducing power (K-salt) 82 per cent of *d*-fructose.

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The Influence of Histidine on the Urinary Excretion of Nitrogen in Dogs Given Pure Amino Acid Mixtures Intravenously[1]

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Introduction

During the course of experiments on the utilization of intravenously administered amino acid mixtures a number of observations were made on the influence of histidine when omitted from an otherwise complete mixture of essential amino acids. These observations were so different from similar experiments in which tryptophan was absent as to indicate a different behavior in the metabolism in these two essential amino acids.

Previous Work

Although no data apparently have been published on the utilization of mixtures of pure amino acids administered intravenously, there are conflicting reports concerning their administration by mouth. Rose (1), in a preliminary note, stated that the requirements for nitrogen balance in adult dogs were the same as the requirements for growth in growing rats, although no detailed data were offered. In nitrogen balance experiments on dogs, Nielsen, Gerber, and Corley (2) found that when mixtures of all of the essential amino acids (except threonine) were used, complete retention of the ingested nitrogen occurred. When single amino acids were used, only cystine, histidine, and lysine seemed to be selectively retained. In experiments on adult white rats Wolf and Corley (3) observed during three day periods that each of the nine amino acids essential for growth was also essential for the maintenance of nitrogen balance. On the other hand, Burroughs, Burroughs, and Mitchell (4), in adult rats, found that positive nitrogen balance could

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be achieved for periods as long as five days with omission of four of the essential amino acids, *i.e.*, lysine, leucine, histidine, and phenylalanine. In previous experiments from this laboratory (5) tryptophan added to an acid hydrolyzate of casein lacking it was shown to influence immediately the urinary output of nitrogen; its effect on nitrogen balance is a fact about which there seems to be general agreement. In the present experiments, histidine was studied in a similar way and while positive nitrogen balance could be achieved for a period of three days, continuation on the histidine deficient mixture led to increased output of urinary nitrogen after this interval.

Methods

Nine experiments were carried out on eight adult female dogs previously prepared with an episiotomy to permit daily catheterizations. They were starved for several days and then given an almost protein-free diet by gavage, consisting of Ringer's solution, Karo syrup, and vitamin B complex (Labco, obtained from the Borden Company). The daily caloric intake was 305 calories and the total nitrogen intake was 0.07 grams per day due to the vitamin supplement. This regime resulted in an excellent diuresis so that between 600 and 900 cc. of urine were collected each day. The usual precautions were observed in collecting and preserving these specimens; catheterization was carried out at each 24 hour period. Fecal nitrogen was measured in a number of instances, but the stools were so scanty that they did not affect the findings significantly and were therefore omitted. Diarrhea was not present. A preliminary period of one to two weeks preceded each experiment in order to achieve as nearly as possible a constant low level of nitrogen output in the urine.

A three or four day preliminary period preceded the administration of the amino acids followed by a second similar period. The amino acids were crystalline and were obtained from Dr. D. F. Robertson through the generosity of Merck and Company. The solution of amino acids were made up according to the formula published by Rose except that in the case of racemic mixtures the unnatural form of which was inactive, the amount was doubled. In Table I is listed the actual composition of this mixture. Solubility was not very great but no great difficulty was experienced in making up a 5 per cent solution; its nitrogen content was 0.7 per cent. The pH of the final solution was adjusted to 6.5 with sodium bicarbonate by adding 10 per cent of the weight of the amino

acids. The solution was sterilized by passing it through a Seitz filter and the calculated daily amount of nitrogen was injected intravenously in six equal doses one hour apart. The amount given, with one exception, was 1.58 grams of nitrogen per day, *i.e.*, about 200 cc.; this represented roughly 25 per cent more nitrogen than appeared in the urine during the preliminary period. When histidine was omitted from the mixture, it was replaced by an equivalent amount of nitrogen as glycine. It will be noted that only essential amino acids were used in the present

TABLE I

Composition of Amino Acid Mixture Used for Intravenous Injection

	Rose (7) mixture (as published minus arginine)	Present mixture	The final 5 per cent solution contained the following (*grams per liter*)
Phenylalanine	0.70	0.70	3.54
Threonine	0.60	1.20	6.06
Leucine	0.90	1.80	9.10
Isoleucine	0.50	1.00	5.05
Tryptophan	0.20	0.20	1.01
Valine	0.70	1.40	7.06
Methionine	0.60	0.60	3.03
Lysine	1.00	2.50	12.63
Histidine	0.40	0.50	2.52
Totals	5.60	9.90	50.0

Note that the mixture as used contains twice as much threonine, leucine, isoleucine, valine, and lysine because these amino acids are active only in their natural form and were available only as racemic mixtures.

Note also that the weights for lysine and histidine in the third column are for their monohydrochlorides.

experiments, with the exception of Experiments 3 and 4, in which a neutralized preparation of an acid digest of casein was used, containing no tryptophan.

Nitrogen determinations were carried out by the method of Sobel, Yuska, and Cohen (6), urea and ammonia by the urease method (8). All measurements were done in duplicate.

Experimental Findings and Comment

In Table II are listed the detailed data obtained during experiments involving the three day periods of injection. It will be noted that the

TABLE II

Total Nitrogen in Urine

The numbers refer to 24 hour periods and are in grams of nitrogen

Experiment	Preliminary period				Injection period			Post-injection period			
	Days										
	1	2	3	4	5	6	7	8	9	10	11
1. Dog 58, 8.1 kg. Rose mixture complete											
Output	1.01	1.29	1.07	1.31	1.14	1.36	2.24	1.09	1.12	1.17	1.25
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	0.07	0.07
Neg. bal.	0.94	1.22	1.00	1.24			0.66	1.02	1.05	1.10	1.18
Pos. bal.					0.44	0.22					
Averages	−1.10				0.00			−1.09			
2. Dog Z3a, 8.2 kg. Rose mixture complete											
Output	1.29	1.21	1.94	1.03	1.18	1.48	1.49	1.71	0.81	—	—
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	—	—
Neg. bal.	1.22	1.14	1.87	0.96				1.64	0.74	—	—
Pos. bal.					0.38	0.08	0.07				
Averages	−1.29				+0.17			−1.19			
3. Dog Z3, 7.0 kg. Acid digest of casein (no tryptophan)											
Output	1.24	1.14	0.92	1.11	1.97	2.49	2.32	1.33	1.49	1.26	1.05
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	0.07	0.07
Neg. bal.	1.17	1.07	0.85	1.04	0.39	0.91	0.74	1.26	1.42	1.19	0.98
Averages	−1.03				−0.68			−1.21			
4. Dog G8, 7.4 kg. Acid digest of casein (no tryptophan)											
Output	1.33	2.05	2.00	1.66	2.13	2.19	2.00	1.77	1.33	1.24	1.10
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	0.07	0.07
Neg. bal.	1.26	1.98	1.93	1.59	0.55	0.61	0.42	1.70	1.26	1.17	1.03
Averages	−1.69				−0.53			−1.29			

TABLE II—*Continued*

Experiment	Preliminary period				Injection period			Post-injection period			
	Days										
	1	2	3	4	5	6	7	8	9	10	11
5. Dog G8, 7.4 kg. Rose mixture minus histidine (replaced by glycine)											
Output	1.77	1.33	1.24	1.10	1.42	1.49	1.59	1.07	—	1.55	0.88
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	0.07	0.07
Neg. bal.	1.70	1.26	1.17	1.03			0.01	1.00	—	1.48	0.81
Pos. bal.					0.16	0.09					
Averages	−1.29				+0.08			−1.09			
6. Dog 58, 8.1 kg. Rose mixture minus histidine (replaced by glycine)											
Output	1.09	1.12	1.17	1.25	1.22	1.50	1.76	1.11	1.33	1.59	1.13
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	0.07	0.07
Neg. bal.	1.02	1.05	1.10	1.18			0.18	1.04	1.26	1.52	1.06
Pos. bal.					0.36	0.08					
Averages	−1.09				+0.08			−1.22			
7. Dog Z16, 7.4 kg. Rose mixture minus histidine (replaced by glycine)											
Output	1.04	1.00	0.93	1.18	1.05	1.35	1.17	1.00	0.82	1.10	1.04
Intake	0.07	0.07	0.07	0.07	1.58	1.58	1.58	0.07	0.07	0.07	0.07
Neg. bal.	0.97	0.93	0.86	1.11				0.93	0.75	1.03	0.97
Pos. bal.					0.53	0.23	0.41				
Averages	−0.97				+0.39			−0.92			

complete Rose mixture led to a positive nitrogen balance in two of the three days in Experiment 1, and during the entire period of Experiment 2. The acid hydrolyzate of casein without tryptophan by contrast produced a marked negative nitrogen balance in each of the six days in Experiments 3 and 4, although there was evidence of some retention as shown by the fact that the negative balance was lower during the injection period.

On the other hand, omission of histidine from the Rose mixture led to a positive balance in each of the three day intervals of Experiments 5, 6, and 7. On the basis of these last experiments, it might be assumed that histidine was unnecessary to maintain positive nitrogen balance. However, we felt that histidine originating from tissue protein breakdown might be utilized more economically than tryptophan and that the body could retain it for several days, but not necessarily indefinitely. At the suggestion of Dr. Carl Cori we therefore prolonged the period of observation for three to nine days, with the results shown in Table III.

TABLE III

Total Nitrogen in Urine

The numbers refer to 24 hour periods and are in grams of nitrogen

Experiment	Preliminary period				Injection period									Post-injection period		
	Days															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
8. Dog J7, 6.8 kg. Rose mixture minus histidine (replaced by glycine)																
Output	0.47	0.52	1.60	1.56	1.07	1.13	1.07	2.00	2.03	2.09	2.86	1.41	1.41	1.29	0.86	0.76
Intake	0.09	0.09	0.09	0.09	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37	0.09	0.09	0.09
Neg. bal.	0.38	0.43	1.51	1.47				0.63	0.66	0.72	1.49	0.04	0.04	1.20	0.79	0.67
Pos. bal.					0.30	0.24	0.30									
Averages	−0.95				+0.28			−0.67			−0.52			−0.88		
9. Dog J8, 8.2 kg. Rose mixture minus histidine (replaced by glycine)																
Output	0.83	1.35	0.65	0.61	1.72	1.83	1.34	2.05	1.86	1.68	1.86	2.52	1.69	0.99	1.03	0.46
Intake	0.10	0.10	0.10	0.10	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	0.10	0.10	0.10
Neg. bal.	0.73	1.25	0.55	0.51	0.14	0.25		0.47	0.28	0.10	0.28	0.94	0.11	0.89	0.93	0.36
Pos. bal.							0.24									
Averages	−0.76				−0.05			−0.28			−0.44			−0.73		

When the period of injection of the histidine-free mixture is prolonged beyond three days, it can be seen from Table III that the output of nitrogen in the urine increases and that from the third day on there is a pronounced negative balance, thus indicating that histidine is really necessary to achieve nitrogen balance with an otherwise complete mixture.

One may infer from the present and other observations that there is a decisive difference in the behavior of tryptophan and histidine as far as their influence on nitrogen balance is concerned. It would appear

that tryptophan before liberation from tissue protein may be in part already metabolized and hence of less value for repair or if liberated as tryptophan is more rapidly metabolized than histidine. Histidine, on the other hand, is apparently used more economically and when released from tissue protein breakdown can be retained for at least three days and utilized in filling out an otherwise complete mixture. However, it cannot do so indefinitely and must therefore be considered as essential

TABLE IV

Urea and Ammonia Nitrogen in Urine

The numbers except where indicated refer to 24 hour periods and are in grams of nitrogen

Experiment	Preliminary period			Injection period									Post-injection period		
	Days														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
8. Dog J7. Urea and ammonia determinations															
Total N	0.52	1.60	1.56	1.07	1.13	1.07	2.00	2.03	2.09	2.86	1.41	1.41	1.29	0.86	0.76
Urea N	0.12	1.03	0.95	0.32	0.28	0.26	0.90	0.75	0.87	1.27	0.33	0.36	0.67	0.51	0.37
NH_3 N	0.16	0.28	0.34	0.18	0.34	0.18	0.11	0.32	0.23	0.15	0.36	0.23	0.19	0.16	0.09
Urea plus NH_3 N	0.28	1.33	1.29	0.50	0.64	0.44	1.01	1.07	1.10	1.42	0.69	0.59	0.86	0.67	0.46
% of total N	54	83	83	47	57	41	51	52	53	50	49	42	67	78	61
Averages (%)	73			48			52			47			69		
9. Dog J8. Urea and ammonia determinations															
Total N	1.35	0.65	0.61	1.72	1.83	1.34	2.05	1.86	1.68	1.86	2.52	1.69	0.99	1.03	0.46
Urea N	0.70	0.12	0.10	0.89	0.73	0.51	1.04	0.67	0.43	0.33	0.98	0.45	0.39	0.50	0.16
NH_3 N	0.12	0.23	0.23	0.16	0.18	0.15	0.16	0.26	0.25	0.33	0.21	0.16	0.30	0.23	0.16
Urea plus NH_3 N	0.82	0.35	0.33	1.05	0.91	0.66	1.20	0.93	0.68	0.66	1.19	0.61	0.69	0.73	0.32
% of total N	61	54	54	61	50	49	59	50	40	35	47	36	70	71	70
Averages (%)	56			53			50			39			71		

for maintaining positive nitrogen balance for periods longer than three days. Similar differences probably exist in other amino acids as far as their ability to be reutilized by the body when released from tissue protein broken down during the course of normal endogenous metabolism. Differences between our findings and those of others (4) may be due to differences in the animal used and in the manner of administration.

Of interest are the urea and ammonia determinations which were

carried out in Experiments 8 and 9 and are shown in Table IV. In Experiment 8 the total urea and ammonia nitrogen represented an average of 73 per cent of the total urinary nitrogen excreted during the three days preceding injection and 69 per cent during the three days following injection. However, during the nine days the pure amino acid mixture was injected, the urea nitrogen plus ammonia nitrogen was at a lower and fairly constant level, averaging 49 per cent of the total urinary nitrogen. In Experiment 9 such marked differences were not apparent; nevertheless, the percentage did fall during the nine days of injection. That the undetermined nitrogen was not due to an excessive loss of amino acids in the urine per se follows from many studies in this laboratory with hydrolyzed protein in which the same rate of injection was used. Only when much larger doses are injected did we observe any evidence of their excretion in the urine. However, it should be pointed out that the pure amino acids used in these experiments contained a large (43 per cent) proportion of unnatural forms inasmuch as many were synthetic racemic mixtures (see Table I). That these unnatural amino acids may be excreted selectively in the urine is an interesting possibility which is being investigated.

Summary

When histidine is omitted from intravenously administered mixtures containing all of the other essential amino acids, positive nitrogen balance is achieved for three days in the dog. After this period urinary excretion of nitrogen increases markedly, leading to a negative balance. It would seem, therefore, that this amino acid behaves quite differently quantitatively from tryptophan, the absence of which from an otherwise complete mixture is reflected immediately by an increased urinary output of nitrogen.

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The Rate of Liberation of Cystine from Proteins by Acid Hydrolysis[1]

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Introduction

In general the rate of hydrolysis of proteins has been measured by the estimation of amino groups. Vickery (1) for example determined the rate of liberation of amino nitrogen from gliadin following hydrolysis with varying strengths of HCl and H_2SO_4. He found that with 20 per cent HCl hydrolysis was practically complete within 20 hours. With weaker acids the time required for complete hydrolysis was progressively greater. Dunn (2) hydrolyzed casein with H_2SO_4 and followed the liberation of amino nitrogen until it reached its maximum value at the end of 24 hours. Greenberg and Burk (3) hydrolyzed gelatin, gliadin, and silk fibroin with both HCl and H_2SO_4 at varying concentrations and for different periods of time. They found that the hydrolysis of these three proteins as measured by the increase in amino nitrogen was a second order reaction and that the catalytic effect of acids on protein hydrolysis is proportional to the thermodynamic activity of the hydrogen ion. Subsequently Nasset and Greenberg (4) showed the acid hydrolysis of casein was also a second order reaction.

Another approach to the rate of hydrolysis of a protein was made by Rimington (5) who followed the liberation of a single amino acid cystine. Using 20 per cent HCl as the hydrolytic agent, Rimington determined the cystine content of wool hydrolyzates by the Sullivan (6) method and found a gradual increase in liberated cystine with time of hydrolysis up to 7 hours. When the Folin-Looney cystine method (7) was used the cystine values did not show a gradual increase as the hydrolysis proceeded but reached a maximum as soon as the wool was dissolved. A similar

[1] A preliminary report of this work was given before the Division of Biological Chemistry, American Chemical Society, Boston, Mass., September 1939.

experiment was conducted by Jones and Gersdorff (8) with casein hydrolyzed by 20 per cent HCl and with estimation of the liberated cystine by the Sullivan colorimetric method. They found a gradual liberation of cystine up to 6 hours hydrolysis. For the next 18 hours the value remained constant. Previously Sullivan (9) had reported that 6 hours hydrolysis of casein gave the maximum cystine value.

In hydrolyzing proteins for amino acids it is desirable to obtain the maximum yields in as short a time as possible and to hydrolyze with the minimum amount of humin formation. With the use of 20 per cent HCl containing $TiCl_3$ Sullivan and Hess (10) found that various proteins could be hydrolyzed with the formation of little humin and with a shortening of the time of hydrolysis to 1 to 2 hours. Though satisfactory in our hands the titanous chloride treatment has some disadvantages in that it is necessary to precipitate the titanium and to wash the more or less colloidal titanous hydroxide precipitate carefully. A more convenient procedure is that of Baernstein (11, 12) who hydrolyzed protein with 57 per cent HI containing KH_2PO_2.

Baernstein's procedure of hydrolysis we found satisfactory. Accordingly we made a study of it in comparison with 20 per cent HCl and with a mixture of HCl and HCOOH found by Miller and duVigneaud (13) to be excellent in the study of the cystine content of insulin. These three hydrolytic acids were then made use of in a study of their relative efficiency in liberating cystine from various proteins. Since both the Folin-Looney (7) and the Okuda (14) methods give positive findings with bound cystine the Sullivan cystine method must be employed in determining the rate of liberation of cystine.

Experimental

Proteins

Six proteins varying considerably in their cystine content were chosen for the experiments. The wool was a carefully prepared sample supplied by Dr. M. Harris of the Textile Foundation, National Bureau of Standards. The finger nail clippings from one individual were cut into small pieces and aliquots were used for the various analyses. The lactalbumin, labeled No. 7HAAX, was supplied by the Borden Company. The gliadin, edestin, and alpha globulin of the lima bean were furnished by Dr. D. Breese Jones of the Bureau of Agricultural Chemistry and Engineering, U. S. Dept. of Agriculture. All the results upon these proteins are expressed on the moisture and ash free basis.

Hydrolysis with 20 per cent HCl

The sample, 50–200 mg. was hydrolyzed in a 30 cc. pyrex flask fitted with a ground glass jointed reflux condenser with 2.0 ml. of 20 per cent HCl in an oil bath at 125–130° for the specified length of time. The temperature of the hydrolyzing solution was 108–110°C. The hydrolyzate was brought to pH 3.5 by the careful addition, with stirring, of $5N$ NaOH and then made to a definite volume with $0.1N$ HCl.

Hydrolysis with HCl-HCOOH

To the sample, 50–200 mg., were added 2.0 ml. of conc. HCl and 2.0 ml. of 95 per cent HCOOH and the mixture was heated in an oil bath at 125–130°C. under reflux condenser for the specified length of time in the pyrex flask. The hydrolyzate was concentrated on a water bath to a syrup which was then dissolved in a definite volume of $0.1N$ HCl.

Hydrolysis with HI

The same sized sample as above was hydrolyzed with 5.0 ml. of 57 per cent HI containing 1.0 per cent KH_2PO_2. Care must be taken to insure the use of an HI solution that is perfectly colorless. The hydrolysis was conducted in an oil bath held at 135–140°C., the temperature of the hydrolyzing solution was 127°C. When the period for hydrolysis was over the HI was removed by allowing it to boil off with the reflux condenser removed. This step took about 30 minutes. The residue was then dissolved in the proper volume of $0.1N$ HCl.

Cystine Determination

The hydrolyzates from the 20 per cent HCl and the HCl-HCOOH methods contained cystine but no cysteine. The hydrolyzates from the 57 per cent HI procedure contained cysteine but no cystine. The Sullivan methods for cystine and cysteine respectively were employed using cystine as the standard in the first instance and cysteine hydrochloride in the second. In the cystine method the NaCN employed was dissolved in N NaOH. The Okuda (14) method which like the Folin-Looney (7) will respond to bound cystine was also used. The results with the Sullivan method are given in Table I with the cysteine values by the HI method expressed as cystine to put all results on the same basis. The values with the Okuda method reached their maximum with each protein and with each method of hydrolysis within two hours and therefore are not included in the table. For every protein there was good agreement

between the maximum value by the Sullivan method and the Okuda values.

Discussion

In the case of the hydrolysis with HCl-HCOOH and with HI at least 30 minutes further heating was needed to drive off the HCl-HCOOH and the HI-H_3PO_2 so the values for 15 minutes and 30 minutes cannot be

TABLE I

Rate of Hydrolysis of Protein

Per cent of cystine liberated with 20 per cent HCl (A), HCl-HCOOH (B), and 57 per cent HI (C)

Protein		Time of hydrolysis, *Hours* 0.25	0.50	1	2	4	6	17	24
Wool	A	4.54	6.48	8.68	12.60	12.66	12.82	12.86	12.65
	B	2.18	2.84	3.14	7.56	10.80	12.53	12.82	12.82
	C		10.80		11.95	12.82	12.76		
Finger nails	A			10.73	12.49	13.62	13.77	13.77	13.71
	B			5.10	7.49	11.83	12.85	13.63	13.71
	C		10.97		12.49	13.87	13.87		
Lact-albumin	A	2.22	2.76	2.99	3.47	3.54	3.71		3.59
	B	1.23	1.53	2.58	2.91	3.54	3.83		3.61
	C		2.96		3.17	3.59	3.59		
Gliadin	A		1.33	1.42	1.65	1.91	1.94	1.88	1.88
	B		0.94	1.18	1.44	1.52	1.85	1.87	1.87
	C		1.48		1.77	1.91	1.90		
Edestin	A		1.01	1.03	1.01	1.19	1.22		1.17
	B		0.89	0.94	0.94	1.13	1.16		1.21
	C		1.03		1.08	1.13	1.21		
Alpha glob. lima bean	A		1.01	1.05		1.44		1.44	1.50
	B		0.70	0.85		1.12		1.25	1.47
	C		0.96	1.11		1.33	1.48		

taken as an accurate comparison with similar values on the 20 per cent HCl hydrolysis which was speedily cooled and worked with after the specified time of heating in the oil bath. In B and C of Table I the actual time of hydrolyzing is slightly longer than given in the table. In general the cystine values reach their maximum with the 57 per cent HI hydrolysis within 4 hours, with 20 per cent HCl within 6 hours, and with the HCl-HCOOH hydrolysis within 17 hours. For several of the proteins, times intermediate between the 6 and 17 hours hydrolysis with

HCl-HCOOH were run with the values becoming progressively greater up to about 17 hours. However, what ever the method of hydrolysis the maximum values by all three methods for each protein are practically identical. In the case of the HI hydrolysis, no humin is formed, with both the HCl-HCOOH and the 20 per cent HCl some humin is formed, but evidently there is no accompanying loss of cystine. Hydrolysis with either 57 per cent HI or 20 per cent HCl is more rapidly accomplished and the analysis can be completed more quickly than with the HCl-HCOOH method. The HI procedure in fact is by far the speedier procedure of hydrolysis and gives the maximum yields in the shortest time without humin formation. It has disadvantages in that the HI and H_3PO_2 must be driven off before testing for cysteine, and the hydrolyzate contains only cysteine and prevents the separate estimation of cystine and cysteine. The HCl hydrolysis has an advantage in that with its use it is possible to study both the cystine and cysteine in the hydrolysis and to estimate methionine colorimetrically.

Cystine Peptides in the HCl Hydrolyzate

There is some evidence of the presence of labile cystine peptides in the acid hydrolyzate with the shorter periods of time. Thus when 5 per cent aqueous NaCN is used in place of 5 per cent NaCN in *N* NaOH in the Sullivan method the cystine values are always less. For example with wool hydrolyzed for 15 minutes with 20 per cent HCl and using aqueous NaCN to split the disulfide bond the cystine value uncorrected for moisture and ash was 2.8 per cent while when the NaCN in *N* NaOH was used the value was 3.9 per cent. Apparently the alkalinity of the cyanide is producing further hydrolysis of a cystine peptide in the hydrolyzate. As the hydrolysis nears completion, however, this difference between the aqueous and the alkaline NaCN disappears until finally both procedures give idential values. With edestin, the alpha globulin of the lima bean, gliadin, and lactalbumin the Okuda cystine procedure gives the same cystine value in 30 minutes hydrolysis as it does in 22 hours, and finger nails give the maximum value in the Okuda method in one hour. It would seem that in these proteins the cystine exists only as a disulfide, S tied to S as RS-SR. In the case of wool, however, the cystine by the Okuda method progressively gets greater with time of hydrolysis from 15 minutes to 3 hours, while in the Sullivan method the cystine increases gradually up to the 7th hour. It would seem that the sulfur in the wool is tied in more than one way as RS-SR

and as RSR′. The possibility that in wool the sulfur is tied in at least two forms will be given a detailed investigation.

Order of Reaction

An attempt was made to use the data on the hydrolysis of wool with 20 per cent HCl to calculate the reaction constants for first and second order. However, the speed of hydrolysis is so great that only a few values

TABLE II

Cystine Liberated from Wool by Hydrolysis with 5 and 10 per cent HCl

Sullivan method-5 per cent aqueous NaCN

Time	Per cent cystine	
	5 per cent HCl	10 per cent HCl
hours		
0.5		1.79
0.75		2.83
1.00	1.29	2.88
1.50		4.58
1.75		5.39
2.00	2.30	5.84
3.00	3.12	6.74
4.00	3.78	7.95
5.00	4.13	8.39
6.00	4.64	9.06
7.00	5.23	10.74
8.00	5.91	11.89
9.00		12.12
10.00	6.76	12.24
13.00		12.19
17.00	8.65	
22.00	8.95	
24.00	9.18	
36.00	9.90	
48.00	12.33	
54.00	12.18	

could be obtained and these seemed to indicate a second order reaction. To test this point further two series of hydrolyses were run with 10 per cent HCl and with 5 per cent HCl. The cystine was determined using aqueous 5 per cent NaCN. Table II gives the cystine values so obtained, corrected for moisture and ash. Table III gives the reaction constants (K) calculated from these data and also the three K values calculated from the data on the 20 per cent HCl hydrolysis. The constants give rather good agreement for a second order reaction up to the time of about

90 per cent liberation of cystine, from this point, as pointed out by Greenberg and Burk (3) the nature of the equation is such that but little reliance can be placed upon the constant. The values of the constant computed for the first order reaction decrease with increased time indicating that the liberation of cystine cannot be a reaction of this order, while the relative constancy of the second order reaction values would place the hydrolysis in the second order of reaction. This finding with wool is in agreement with the findings of Greenberg and Burk (3)

TABLE III

Values of K Calculated for First and Second Orders of Reaction for Three Concentrations of HCl

Time	20 per cent HCl		10 per cent HCl		5 per cent HCl	
	First order	*Second order*	*First order*	*Second order*	*First order*	*Second order*
hours						
0.25	0.71	0.015				
0.50	0.60	0.020	0.137	0.00345		
0.75			0.152	0.00401		
1.00	0.48	0.020	0.117	0.00308	0.0481	0.00117
1.50			0.135	0.00398		
1.75			0.147	0.00434		
2.00			0.141	0.00456	0.0448	0.00115
3.00			0.115	0.00408	0.0422	0.00113
4.00			0.114	0.00463	0.0397	0.00111
5.00			0.100	0.00418	0.0333	0.00100
6.00			0.098	0.00474	0.0341	0.00101
8.00					0.0354	0.00115
10.00					0.0345	0.00121
17.00					0.0313	0.00138
22.00					0.0254	0.00102
24.00					0.0247	0.00120
36.00					0.0196	0.00113
Average............		0.018		0.00404		0.00113

for gelatin, gliadin, and silk fibroin, and with that of Nasset and Greenberg (4) for casein.

It has been suggested by Greenberg and Burk (3) that the catalytic effect of acids on the rate of hydrolysis is proportional to the thermodynamic activity of the acid rather than to the concentration of the acid. Employing the average velocity constant for the second order of reaction for each of the three concentrations of the acid and dividing by the activity coefficients for each concentration as given by Lewis and Randall (15) the following values were obtained; $K/a^{\pm}$ 20 per cent HCl 0.00116,

10 per cent HCl 0.00116, and 5 per cent HCl 0.0009. The agreement between these values tends to confirm the suggestion of Greenberg and Burk (3), and also is further evidence that the reaction is of the second order.

Summary

Six proteins varying considerably in their cystine content were hydrolyzed by HCl, HCl-HCOOH, and HI (H_3PO_2) for periods varying from 15 minutes to 24 hours, and the cystine liberated was estimated by the Sullivan method which is positive only with free cystine. The maximum values by all three procedures of hydrolysis for each protein are practically identical. In the case of HI hydrolysis no humin is formed while with 20 per cent HCl and with the HCl-HCOOH some humin is formed but with no loss of cystine. Hydrolysis with HI or 20 per cent HCl is more rapidly accomplished than with the HCl-HCOOH procedure. The HI procedure has a disadvantage in that HI and H_3PO_2 must be driven off before testing for cysteine and cannot be used where the separate estimation of cystine and cysteine is desired. The slightly slower HCl hydrolysis has an advantage in that with its use it is possible to study both the cystine and cysteine in the hydrolysis and to estimate methionine colorimetrically. There are indications that in wool the cystine is tied in more than one form. Employing the data gathered on hydrolysis with 5 and 10 per cent HCl the liberation of cystine from wool is a second order reaction.

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Study of Various Chemicals Depressing the Thyroid Gland

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Introduction

Investigative effort has indicated a common origin of three general types of reaction formerly regarded as independent. Cutting and Kuzell (1) first demonstrated in guinea pigs the activity of sulfanilamide in lowering the basal metabolic rate of animals treated with the thyrotropic hormone. Histologically, they observed that sulfanilamide-treated glands showed only the slightest degree of hyperplasia as compared with the marked hyperplasia in controls. The conclusion was that sulfanilamide and the other effective chemical agents depressed the thyroid epithelium.

Mackenzie, *et al.* (2, 3) noted thyroid hyperplasia and hypothyroidism associated with the feeding of sulfaguanidine in the diet. This reaction occurred in animals on stock diets to which were added sulfonamides, sulfadiazine, sulfapyridine, and sulfanilamide being effective. The latest report of these workers (4) demonstrated that sulfonamides and thiourea were effective in the production of this syndrome. Similar results were reported by Astwood, *et al.* (5). Kennedy (6) first noted the action of thioureas in producing enlarged thyroids. Astwood (7) recently presented evidence of the efficacy of thiouracil and of thiourea in the treatment of hyperthyroidism in humans, and referred to a paper (14) in which the effect is demonstrated in animals with 2-thiouracil, 2-thiobarbituric acid, diethyl thiourea, and several derivatives of 2-thiohydantoin. He also refers to a number of aniline derivatives, *p*-aminobenzoic acid and *p*-aminophenylacetic acid.

Studies of the "potentiation" of insulin by various agents (8, 9) have demonstrated the activity of saccharin, benzenesulfonamide, sulfadiazine, sulfaguanidine, sulfathiazole, sulfanilamide, taurine, *p*-, *o*-aminobenzoic acids, thiosalicylic acid, ephedrine and β-alanine.

Thus, three lines of investigation suggest a common origin and that an agent capable of acting in one system is also capable of acting in the others. To prove the contention that all three are interchangeable, it was decided to take a typical member of each chemical group reported effective and try it in the other two systems and to check it in the original reaction. Potentiation or pseudo-potentiation of the action of insulin in starved mice was used as one criterion; production of hypertrophied thyroids in rats as the second, and reduction of thyroxin activity in its effect of hypocholesteremia in dogs as the third. The agents selected were *p*-aminobenzoic acid, reported (9) active in "potentiating" insulin; sulfaguanidine, reported (2, 3, 4, 5) active in producing thyroid hypertrophy; sulfanilamide reported (1) active in counteracting thyroxin activity; and finally, thiourea, reported (4, 5, 6) active in producing hypertrophied thyroids.

Results

p-Aminobenzoic Acid

p-Aminobenzoic acid in large doses (1 mg. per gram) was non-toxic in normal mice, starved or not starved. This dosage of the aromatic amine potentiated the action of insulin, which, when given in dosages of 0.006 unit per gram, caused 60% convulsions and deaths. Insulin alone in this dosage will not produce any reaction of a shock-like character. There is no potentiation of insulin in non-starved mice by *p*-aminobenzoic acid under any conditions. It must be remembered that starved mice are generally more susceptible to toxic action of any chemical and that *p*-aminobenzoic acid is therefore toxic, producing some 50% deaths in mice at levels of 2 mg. per gram, whereas in non-starved animals the toxic level or LD 50 is 6 mg. per gram. *p*-Aminobenzoic acid produces no insulin potentiation at levels of 0.5 mg. per gram, in animals starved approximately 24 hours.

p-Aminobenzoic acid increased the action of sulfonamides in producing hypertrophied thyroids (confirming the conclusion of Mackenzie, *et al.* (4)) and was effective alone. Stock diets and synthetic diets supplemented by 1.0 and 2.0% *p*-aminobenzoic acid produced in rats in two to three months thyroids which averaged 22 mg. per 100 grams, contrasted to the normal value of 8 to 10 mg. Sulfaguanidine was approximately as active as *p*-aminobenzoic acid, producing in a similar period of time thyroids weighing 20 to 30 mg. per 100 grams. Relatively, therefore, *p*-aminobenzoic acid was as potent as the sulfonamides in producing thyroid enlargement.

Finally, according to the technique of Cutting and Kuzell (1) the effect of *p*-aminobenzoic acid in counteracting the action of thyroxine was checked, using the degree of hypocholesterolemia and survival times as the indices. Thyroxine was given at two levels, namely, 0.1 mg. per each 350 gram guinea pig and at a level of 100 micrograms per each kilogram of dog. The higher level results in death of 100 per cent of the guinea pigs so treated within 2 weeks, making it possible to use prolonged survival period as an index of the ability of any chemical agent to counteract thyroxine. The use of hypocholesterolemia as an index, using smaller dosage of thyroxine in dogs, was equally effective in demonstrating the anti-thyroid activity of *p*-aminobenzoic acid. The total cholesterol level in the blood of 6 normal dogs averaged 200 mg. per 100 ml., whereas when treated for two weeks with thyroxine the level is 160 mg. per 100 ml. Simultaneous treatment with thyroxine and *p*-aminobenzoic acid at 1 mg. per gram resulted in levels of 185 mg. per 100 ml. (average for six dogs), indicating the anti-thyroid activity of this compound. All of a control set of 20 guinea pigs given 0.1 mg. of thyroxine daily subcutaneously died within two weeks, whereas in the experimental group given the same amount of thyroxine and 1 mg. per gram daily of *p*-aminobenzoic acid orally, only one out of ten died in this period. Thus, *p*-aminobenzoic acid counteracts the action of thyroxine, produces hypertrophied thyroids when fed in the diet and seemingly potentiates the action of insulin.

Sulfaguanidine and Sulfanilamide

Sulfaguanidine and sulfanilamide, as reported (8), were both found active in potentiating the action of insulin, the degree being indicated in Table I. Both were also found to be effective agents in the production of hypertrophied thyroids (Table I). Finally, the ability of both of these agents to counteract the activity of thyroxine is shown in Table I, in which are listed results given as cholesterol levels and survival periods.

Thiourea

As in the case of *p*-aminobenzoic acid and the sulfonamides, thiourea was found effective in potentiating insulin action, producing hypertrophied thyroids and counteracting the activity of thyroxine (Table I). This leads to the conclusion that in all three cases the action is due to an inhibition or depression of the thyroid activity. In Table I is presented a list of compounds tested for two or more of the activities under

consideration. Relatively, the activities for the various chemical agents are the same.

In Table II the compounds are listed which have been demonstrated to potentiate the action of insulin, an action due to thyroxine or thyroid inhibition. It is predicted that each compound effective in one system will be effective in each of the others.

Rats showing marked hypertrophy of the thyroids manifest no alterations in the level of blood cholesterol, which is usually elevated in the blood of dogs receiving sulfaguanidine containing diets. The blood lipoid picture in dogs with demonstrated thyroid hypertrophy maintained for six months on synthetic diets containing 2% of sulfaguanidine shows a decrease in the total cholesterol by some 20% and a simultaneous increase in the free cholesterol blood level by from 20 to 50%. One dog autopsied on a day when its blood lipoid picture was as indicated above showed thyroids weighing 98.4 mg. per each 100 grams of body weight, which is approximately ten times the average value of 10.2 mg. This observation is interesting in view of the findings of Daft, *et al.* (13) which indicate arterial calcification in rats fed sulfaguanidine. The possibility is that altered thyroid status precipitated by any of the chemical agents under consideration may bring about such a disturbance

TABLE I

Anti Thyroid Activity of Various Compounds as Manifested in

Compound	Thyroid weights in mg./100 grams of rat on diets containing compound at indicated level	Insulin "potentiation" in starved mice, value represents no. times the potency of insulin has increased	Anti thyroxine activity: Survival period in G.P.'s receiving 0.1 mg. thyroxine daily for 2 weeks	Anti thyroxine activity: Total cholesterol blood levels in dogs on 20 μg. per 200 g. daily mg./100 ml.
			Percentage surviving	2 weeks
p-Amino-benzoic acid	20 ± 5 mg. 1 and 2% diet	8X 1 mg./gram daily	90% 1 mg./gram daily	180 mg. 1% diet
Sulfanilamide	18 ± 5 mg. 0.5% diet	3X 0.5 mg./gram daily	50% 0.5 mg./gram daily	175 mg. 0.5% diet
Sulfaguanidine	30 ± 10 mg. 1 and 2% diet	2X 3.0 mg./gram daily	60% 3.0 mg./gram daily	180 mg. 1 and 2% diet
Thiourea	30 ± 10 mg. 1% diet	4X 1.0 mg./gram daily	80% 1.0 mg./gram daily	195 mg. 1% diet
Thiobarbituric acid	30 ± 10 mg. 1% diet	5X 1.0 mg./gram daily	90% 1.0 mg./gram daily	—
Normal	10 ± 2 mg.	1X	0%	200 mg.

in the blood cholesterol picture as to result in colloidal instability with consequent deposition of the cholesterol in the blood vessel walls. It may be that experimental conditions have not been quite right to detect

TABLE II

A List of Compounds Tested for Action in Producing a Pseudo-Insulin Potentiation in Starved Mice

Compound	Dosage	Activity	Compound	Dosage	Activity
p-Aminobenzoic acid	1 mg./g.	+	Saccharin	1 mg./g.	+
o-Aminobenzoic acid	0.5 mg./g.	+	Benzenesulfonamide	2 mg./g.	+
m-Aminobenzoic acid	1 mg./g.	+	Acetylsalicylic acid	1 mg./g.	+
p-Hydroxybenzoic acid	0.25 mg./g.	−	Thiourea	0.5 mg./g.	+
o-Aminobenzenesulfonic acid	1 mg./g.	−	Methylthiourea	0.5 mg./g.	+
m-Aminobenzenesulfonic acid	1 mg./g.	−	*S*-Methyl isothiourea sulfate	0.5 mg./g.	+
Sulfanilic acid	2 mg./g.	−	Urethane	2.0 mg./g.	−
Phthalic acid	2 mg./g.	−	Ascorbic acid	0.5 mg./g.	−
Salicylic acid	1 mg./g.	+	Cysteine	2.0 mg./g.	−
Sulfosalicylic acid	0.2 mg./g.	−	Taurine	2.0 mg./g.	+
Aniline	1 mg./g.	−	Cystine	2.0 mg./g.	−
o-Aminophenol	0.25 mg./g.	+	2 - (1 - Piperidylmethyl)-1, 4 - benzodioxan	10.0 mg./kg.	−
Phenol	0.25 mg./g.	−	Phloridzin	1.0 mg./g.	−
Thiosalicylic acid	0.5 mg./g.	+	Atophan	0.5 mg./g.	++
Benzoic acid	1 mg./g.	−	*β*-Alanine	2 mg./g.	+
p-Aminophenylacetic acid	1 mg./g.	+	Glycine	2 mg./g.	−
Sulfanilamide	2 mg./g.	+	*α*-Alanine	2 mg./g.	−
Sulfaguanidine	2 mg./g.	+	Adrenaline	0.5 cc. 1:10,000	−
Sulfapyridine	2 mg./g.	+	Tyramine	1 mg./g.	−
Sulfathiazole	2 mg./g.	+	Ephedrine	0.02 mg./g.	+
Catechol	0.25 mg./g.	−	Alloxan	0.5 mg./g.	+
p-Phenylenediamine	0.1 mg./g.	−	Thiouracil	1 mg./g.	+
o-Cresol	0.25 mg./g.	−	Potassium thiocyanate	1 mg./g.	+
p-Nitrobenzoic acid	1 mg./g.	+	Thiobarbituric acid	2 mg./g.	+
Hippuric acid	2 mg./g.	±	Sodium bicarbonate	2 mg./g.	−
Dopa	2 mg./g.	−	Ammonium chloride	1 mg./g.	±

the altered lipoid picture in the rat. This is in keeping with the fact that thyroidectomy in the rat does not alter the blood lipoid picture, while it does alter the lipoid picture in nearly all other animals.

Rats maintained for 6 months on a synthetic diet containing 2 per cent

sulfaguanidine and then placed on a stock diet without sulfonamide for one month show little if any regression in the hypertrophy of the thyroid glands.

Discussion

In the series of compounds disclosed by Macallum and Sivertz (8), the property of potentiating the action of insulin is not confined to the aromatic aminosulfonic acids inasmuch as taurine, which is an aliphatic aminosulfonic acid, is the most effective agent. In fact, this report would indicate that amino sulfonamides and aromatic sulfonamides also possess this unique property. The work here reported extends the list of effective compounds. The aminobenzoic acids are all effective; aniline and benzoic acid are negative. The property, therefore, does not reside in either the amino or the carboxyl radical in itself, but is a combination of both and has no specific structural relationship on the benzene ring, as all isomers are active, ortho isomer being the most active. In the aliphatic series, the α-amino-carboxylic acids are not effective. However, the β-amino-carboxylic acid tried was effective in some degree. The activity is found in aminophenol but not in phenol itself. The amino group is apparently not vital, as demonstrated by the ability of salicylic acid to replace it. Here again, the ortho compound, salicylic acid, is more effective than the other isomeric hydroxybenzoic acids. The activity of thiosalicylic acid illustrates substitution of the oxygen of the hydroxyl by sulfur to form a thio radical with little loss in potency. Potency seems to decrease from ortho to meta to para in any given effective series, *e.g.*, the aminobenzoic acids. The hydroxyl, carboxyl, amino, sulfhydryl, sulfonamides seem to be interchangeable inasmuch as the aminobenzoic acids, the hydroxybenzoic acids, the aminophenols, thiobenzoic acids, aminosulfonamides are all effective. However, a ring substituted by two carboxyls, two phenolic groups, or two amino radicals, are all negative in their effect under our experimental conditions. The question remains as to what properties characterize molecules of the active type. It is not a specific mechanism but rather a general one associated with a wide variety of organic molecules. It is probable that the suggestion of Cutting and Kuzell (1) is correct in that these various agents are general depressants to the tissues. For a detailed consideration, it is best to consider one chemical and argue the case for that chemical. It seems justifiable to discuss *p*-aminobenzoic acid. The facts to be considered are:

1) P.A.B. acts in the pseudo-potentiation of insulin,
2) produces a mild hyperglycemia in doses of 1 and 2 mg. per gram in dogs,
3) depletes the glycogen reserves of the liver (9),
4) causes a mild rise in blood pressure in the anesthetized cat, reversed by 2-(1-piperidylmethyl)-1,4-benzodioxan,
5) is known to inhibit various enzyme systems (12),
6) produces hypertrophied thyroids with hypothyroidism, and
7) protects against the action of thyroxine in the intact animal.

This type action of P.A.B. is dissociated from its activity as a vitamin. Certain lines of evidence, namely, 2, 3, 4, suggest that the action might be motivated through protection of adrenalin against oxidation, an established point (12). This is ruled out by the failure of adrenalin to bring about this pseudo-potentiation of insulin. Ephedrine, another pressor base, is potent, while tyramine is inactive. The action cannot be simply one of prevention of the formation of thyroxine, as P.A.B. affords protection against the injection of excessive doses of preformed thyroxine. This could be a quantitative decrease through inhibition of thyroid thyroxine formation, but the degree of protection afforded by P.A.B. argues against this conclusion. P.A.B. does not alter the degree of hypoglycemia produced by insulin nor does it alter the duration of the hypoglycemia; thus it is not a factor in maintaining the blood glucose levels. The evidence then suggests that motivation is via the pituitary and production of the thyrotropic hormone, a conclusion reached by Astwood, *et al.* (5) and Mackenzie, *et al.* (4).

Bodansky (10) demonstrated increased sensitivity to insulin in thyroidectomized sheep but found that thyroid administered to thyroidectomized rabbits decreases the hypoglycemic action of insulin. He attributed this action to the ability of thyroid in promoting glycogenolysis. Generalized increase in susceptibility to insulin has been demonstrated in hypothyroidism in human beings (11). The ability of sulfanilamide to counteract thyroxine by depressing the thyroid epithelium has been demonstrated (1). Further, the action of a number of agents, including thioureas, sulfanilamide, and *p*-aminobenzoic acid in producing hypertrophied thyroids (2, 3, 4, 5, 6) by inhibiting the production or liberation of thyroxine has been demonstrated. It is probable that any agent which seems to potentiate the action of insulin in starved mice acts by what could be called a chemical inhibition of thyroid function: a true potentiation of the action of insulin does not occur.

The hypertrophied thyroids associated with hypothyroidism produced by these chemical agents do not regress following discontinuation of the treatment, indicating a danger in their general use; but this remains to be established. In one instance, a chemical active in producing pseudo-potentiation of insulin has been demonstrated (15) to produce extreme thyroid hyperplasia with laboratory signs of hypothyroidism in the human species. The chemical is potassium thiocyanate, used extensively in the treatment of hypertension. *p*-Aminobenzoic acid, which is extremely effective in the production of hypertrophied thyroids, is an agent commonly used in the treatment of gray hair. In the course of treatment it is used over long periods in relatively high doses, 300 or 400 mg. daily which may make it dangerous. More detailed study of human cases is certainly indicated before generalized recommendations are made which might lead to indiscriminate use. This same condition obtains when sulfonamides are used over prolonged periods in the treatment of rheumatic fever. Extreme caution should be observed and patients checked for manifestations of hypothyroidism in all cases. The effectiveness of various of these agents in counteracting hyperthyroidism indicates that more of them should be tried in human beings. Astwood (7) has reported on the efficacy of thiourea and 2-thiouracil in this connection. As potassium thiocyanate is a chemical demonstrated to produce hypothyroidism in man (15) and in view of its previous use in medicine, it is suggested that it is worthy of trial.

Summary

Certain chemicals (thiourea, sulfonamides, *p*-aminobenzoic acid, etc.) are capable of causing three general types of reaction:

a) Pseudo-potentiation of insulin in starved mice,

b) Production of thyroid hypertrophy with hypothyroidism, and

c) Protection against the toxic action of injected thyroxine.

It is suggested that all activities under consideration are due to a generalized inhibition of tissue metabolism. A list is given of 45 compounds tested for one or more of these activities.

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The Genetic Control of Biochemical Reactions in Neurospora: A Mutant Strain Requiring Isoleucine and Valine[1]

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Introduction

X-ray or ultra-violet treatment of the ascomycete *Neurospora crassa* gives rise to "biochemical mutants" that may be identified by the fact that the "minimal medium" capable of supporting the growth of the normal strain will not support the growth of such mutant strains unless accessory growth-factors are added (Beadle and Tatum, 1941). A variety of such mutant strains have been identified (Tatum and Beadle, 1942), and a number of them have been shown to be inherited as though differentiated from normal by single genes. Several have lost the ability to synthesize known vitamins, while in others the ability to synthesize specific amino acids has been lost. The present paper deals with the amino acid requirements of such a mutant strain.

Preliminary Tests

Strain number 16117, derived from a single ascospore produced after x-ray treatment, shows little or no growth on minimal medium, but grows well on complete medium, a medium containing yeast and malt extracts in addition to the components of minimal medium. Single ascospore cultures derived from crosses with the normal strain show segregation such as would be expected if the inability to grow on minimal medium were differentiated from normal by a single gene. (See section on genetic data.)

The growth rate of mutant 16117 on the most favorable media so far found is not entirely equal to the growth rate of the normal strain. This

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sub-normal growth rate and a characteristic clumping of the conidial masses appear to be inherited with its growth-factor requirement. Small inocula of this mutant show no macroscopic growth on minimal medium during a period of 5–6 days. After a longer period, however, growth begins, and measurement in special growth tubes shows the rate ultimately to attain a value almost equal to that on supplemented medium. On media containing the required growth-factor at low concentrations a similar phenomenon is observed with the exception that it becomes adapted more rapidly. Conidia formed by such an "adapted" strain (see section on adaptation) gives rise to cultures that appear to be no different from the original mutant.

TABLE I

Amino Acids Tested Singly and in the Combinations 1–10, 11–23, and 1–23 for Growth Activity on Mutant 16117

1. *dl* Valine	13. *dl* Serine
2. *dl* Isoleucine	14. β-Alanine
3. *dl* Leucine	15. *dl* Norvaline
4. *dl* Threonine	16. *dl* Norleucine
5. *dl* Phenyl alanine	17. *dl* Ornithine
6. *dl* Methionine	18. *dl* Citrulline
7. *l* (−) Histidine	19. *l* (−) Asparagine
8. *dl* Arginine	20. *l* (−) Glutamic acid
9. *l* (+) Lysine	21. *l* (−) Proline
10. *l* (−) Tryptophan	22. *l* (−) Hydroxy proline
11. Glycine	23. *l* (−) Cystine
12. *dl* Alanine	

The mutant grows on medium containing yeast- or malt-extract, but grows equally well on medium containing hydrolyzed casein. The growth effect of hydrolyzed casein or yeast-extract could not be brought about by a mixture of all known amino acids or vitamins. The fact that casein itself has slight activity, while hydrolyzed casein is very active, strongly suggested the essential factor to be an amino acid. As casein itself was slightly active, the possibility that the factor was a vitamin-like contaminant adsorbed on the casein had to be considered. Evidence that the active principle was not a contaminant was the fact that a methyl alcohol-extract of casein is inactive when tested on this mutant. *Neurospora* is known to form extracellular proteolytic enzymes (F.A.F.C. Went, 1901), and thus it would seem reasonable to explain the activity of casein as due to a slow proteolysis. It was, therefore, concluded that

the factor is found in casein and freed by enzymatic or chemical hydrolysis.

Known amino acids were tested for activity singly and in combination. Table I lists the amino acids tested and the combinations used. These 23 amino acids are inactive when tested singly or in two groups of 10 and 13 according to their indispensibility or dispensibility for the rat (Rose, 1938). They are also inactive when all 23 are tested together.

On the basis of this information it was postulated that the factor required by mutant 16117 for normal growth was an heretofore unidentified amino acid.

Bioassay Methods

Three methods have been used to determine the influence of supplements to minimal medium on the growth of mutant 16117. The first of these is designed to give rapid qualitative results for use in following the course of fractionation. It involves making up in minimal media several concentrations of each fraction to be tested, dispensing these media in 10 ml. amounts to 20 × 150 mm. test tubes, autoclaving, inoculating, and incubating at 34°C. for 12-15 hours. The activity of the extract is then estimated by noting the lowest concentration permitting growth and comparing this to growth on minimal medium supplemented with known amounts of hydrolyzed casein.

Quantitative estimations of growth response were made by a second method in which cultures are grown for 50-55 hours at 34°C. in 125 ml. Erlenmeyer flasks, each containing 20 ml. of medium. After incubation the mycelial pad is removed, dried at 100°C. and weighed.

A third method was used in which rates of growth were determined in special tubes (Beadle and Tatum, 1941; and Ryan, Beadle, and Tatum, unpublished) in which progression of a mycelium along an agar surface is measured. This method is particularly useful in studying the growth on media to which the mutant becomes "adapted" over a period of time.

In all cases the minimal medium used was that described by Beadle and Tatum (1941) with the addition of a trace element mixture. This medium has the following composition: NH_4tartrate, 5 g.; NH_4NO_3, 1 g.; KH_2PO_4, 1 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; NaCl, 0.1 g.; $CaCl_2$, 0.1 g.; B, 0.01 mg.; Cu, 0.04 mg.; Fe, 0.20 mg.; Mn, 0.02 mg.; Mo,0.02 mg.; Zn, 0.15 mg.;[2] sucrose, 10 g., biotin (as SMA concentrate #1000), 4 gammas; H_2O, 1 liter. When used in growth tubes this medium is solidified with 3 per cent purified agar.

Fractionation of Casein

The factor required by this mutant was purified by fractionating casein. The enrichment of the active substance was followed by assay-

[2] It is recommended that Zn be increased to 2.0 mg. (see Stokes, Foster, and Woodward, 1943).

ing for it with the mutant. The fractionation scheme shown in Table II yielded a crystalline preparation of high activity (Fig. 1). This crystalline preparation, together with its mother liquor, accounts satisfactorily

TABLE II

Fractionation Carried Out for Purifying the Factor Present in Casein Required by Mutant 16117 for Growth

Treatment	Properties of Active Fraction
Whole casein—refluxed 48 hrs. with concentrated HCl	
HCl removed in vacuo	
Treated with norite	Not adsorbed on norite
Made up to 70 per cent EtOH	Not precipitated
Saturated with CO_2	
Set at 5° C. for 24 hours	Not precipitated
EtOH removed in vacuo	
$CuCO_3$ added in excess and the formed copper salts dried, pulverized, and extracted with water	Copper salt soluble in water
Water soluble copper salts redried and pulverized and extracted with absolute MeOH	Copper salt soluble in absolute MeOH
MeOH soluble copper salts decomposed with H_2S	
Extracted with butyl alcohol at atmospheric pressure	Soluble in hot butyl alcohol, but crystallizes on cooling
Fraction crystallizing from hot butyl alcohol recrystallized from 50 per cent EtOH-H_2O	Crystallizes from 50 per cent EtOH-H_2O

for the observed activity of the starting material. Elementary analysis of the crystalline preparation gave the following results:

	%C	%H	%N
Calculated for $C_6H_{13}O_2N$	54.92	10.01	10.68
Calculated for $C_5H_{11}O_2N$	51.25	9.48	11.96
Observed	52.60	9.59	11.28

The ratios of C, H, and N indicated that this preparation consisted of a mixture of C-5 and C-6 aliphatic, monoamino monocarboxylic amino acids.

Separation of this material into its pure components was found to be extraordinarily difficult. The most useful technique was found to be

fractional crystallization from water-ethanol mixtures. Other means of fractionation were also employed; for example, extensive use was made of the 3,5-dinitrobenzoyl derivative as described by Town (1941) and Saunders, *et al.* (1942). None of the methods used, however, gave active

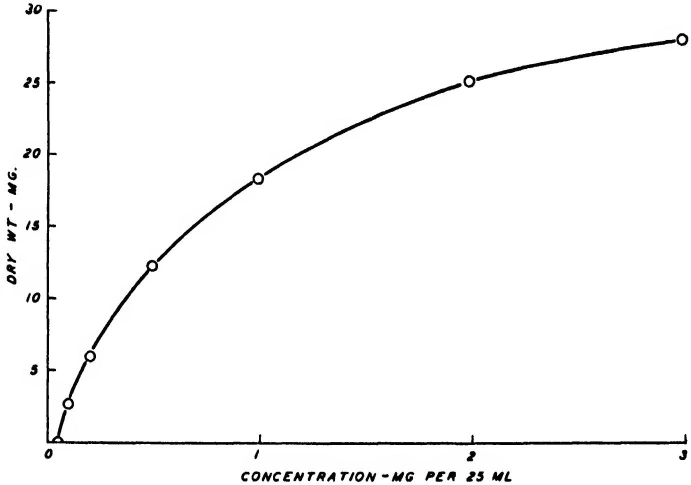


FIG. 1

The Growth Response of Mutant 16117 to a Crystalline Preparation Obtained by Fractionation of Casein

Cultured 55 hours at 34°C.

preparations of unequivocal purity. By means of repeated fractional crystallization a preparation was obtained giving the following analysis:

	%C	%H	%N
Calculated for $C_6H_{13}O_2N$	54.92	10.01	10.68
Observed	54.55	9.75	10.91

The elementary analysis of this preparation indicates that it consists predominantly of a C-6 compound. Considering the difficulties in the separation of the C-5 and C-6 neutral amino acids such a composition would ordinarily be taken as indicating that the active substance in question is a C-6, monoamino, monocarboxylic aliphatic amino acid. The surprising feature of this separation, however, is that despite the great alteration in composition, the product obtained after repeated fractionation from water-ethanol mixtures showed very little increase in

activity. Furthermore, no active preparation was obtained which gave an analysis indicating a pure C-5 or C-6 compound. These facts perhaps might indicate that the activity is due to a mixture of amino acids, and that this activity can be maintained in mixtures with some variation in composition. A mixture of the four aliphatic neutral amino acids, leucine, isoleucine, norleucine, and valine, was retested, and again found inactive. It seemed probable, therefore, that one of the components of the isolated active preparation was an unidentified amino acid. The substances present in the most active preparation were therefore identified.

Properties and Identification of the Material Isolated from Casein

Titration in 80 per cent ethyl alcohol gave an equivalent weight of 130. To check the possibility of the material being a dipeptide, a molecular weight determination was carried out on the 3,5-dinitrobenzoyl derivative using the micro Rast method:

Calculated for 3,5-dinitrobenzoyl C-6 amino acid..	365
Observed for 3,5-dinitrobenzoyl leucine...	360
Observed for 3,5-dinitrobenzoyl substance ..	334

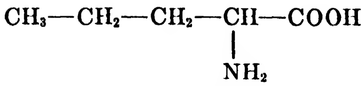
The substance in question must then be represented by the empirical formula $C_6H_{13}O_2N$, and not by a multiple of this.

Measurement of the optical rotation of a preparation containing 15 per cent C-5 (2.3 per cent solution in 20 per cent HCl) gave the following value:

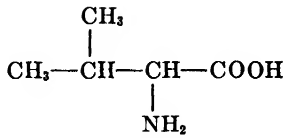
$$[\alpha]_D^{18} = +29.3°.$$

This value indicates either a mixture of amino acids or an unrecognized amino acid. Since treatment of a sample with *d*-amino oxidase showed that no significant racemization had taken place during the isolation procedures, this rotation must be duplicated by any active substance, or mixture of substances.

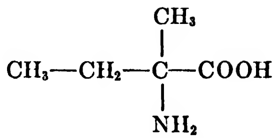
The common methods of degradation were ineffective in elucidating the identity of the substance. Oxidation by use of Chloramine T (Dakin, 1917) yielded carbonyl compounds that could be isolated as the 2,4-dinitrophenyl hydrazones or as *p*-nitrophenyl hydrazones. Melting points of these derivatives indicated the presence of isoleucine plus some contaminant. Quantitative oxidation to the fatty acids proved difficult and was therefore impracticable. The easiest means of checking the



NORVALINE



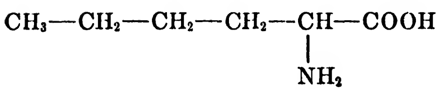
VALINE



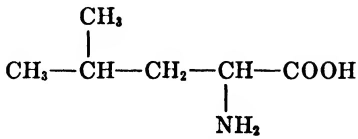
ISOVALINE

FIG. 2

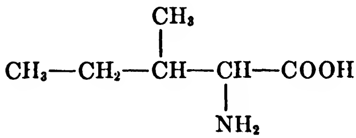
The Three Possible C-5 Amino Acids of Empirical Formula $C_5H_{11}O_2N$



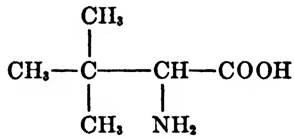
NORLEUCINE



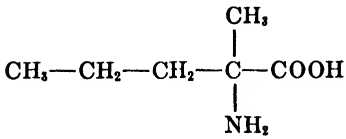
LEUCINE



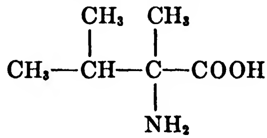
ISOLEUCINE



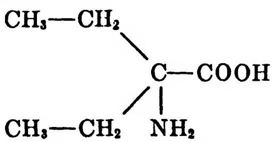
tert.-LEUCINE



α-METHYL-NORVALINE



α-METHYL-VALINE



α-AMINO-DIETHYL-ACETIC ACID

FIG. 3

The Seven Possible C-6 Amino Acids of Empirical Formula $C_6H_{13}O_2N$

identity of the isolated material seemed to be exclusion of the various possible structures, either by testing synthetic material for activity on the mutant or by unambiguous chemical reactions.

All structures with the amino group in the γ, δ, or ϵ position can be eliminated since these do not form copper salts (H. Meyer, 1931) whereas the active isolated material does (Table II). Further evidence that they need not be considered is that the isolated material gives a ninhydrin reaction at dilutions comparable to an ordinary α-amino acid. β-amino structures can be eliminated on the basis of the failure of the isolated product to form a Reineckeate (Dakin, 1933) and the failure of β-amino acids other than β-alanine to give a ninhydrin reaction. Only α-amino structures remained to be considered. The three possible C-5 α-amino structures are shown in Fig. 2. These three were tested and found to be inactive both when tested individually and when tested in the presence of the other natural neutral aliphatic amino acids.

The seven possible C-6 α-amino structures are shown in figure 3. With the exception of α-methyl-norvaline all of these structures were tested singly and in combination with the other neutral aliphatic amino acids. They were found inactive under all conditions tested. α-Methyl-norvaline can be ruled out since while α methyl amino acids give an iodoform reaction, the iodoform reaction on the isolated material was negative.

This evidence indicates clearly that activity of the preparation obtained from hydrolyzed casein is not due to any heretofore unrecognized amino acid. The requirement of this mutant must therefore consist of known amino acids.

Activity of Mixtures of Known Amino Acids

In view of the fact that a mixture of known amino acids must account for the requirement of mutant 16117, all the aliphatic neutral amino acids were tested in all possible combinations, two at a time. In this way it was found that a mixture of 50 per cent *dl*-isoleucine and *dl*-valine is active. All other combinations are inactive. .

Since a mixture of isoleucine and valine is active, but is inactive when tested in the presence of the other essential amino acids, or in the presence of leucine and norleucine, certain amino acids must be strongly inhibitory. This inhibition must be a special attribute of mutant 16117, since wild type grows normally on all mixtures of known amino acids tested. All the amino acids listed in Table I were tested singly for their

effect on the activity of a 50 per cent mixture of *dl*-isoleucine and *dl*-valine. Of these, phenylalanine, norleucine, and norvaline showed inhibitions at concentrations similar to those of the mixtures originally

TABLE III

The Activity of the Isomers of Isoleucine and Valine, Expressed as mgs. Mycelium Dry Weight, After Culturing 55 hrs. at 34°C.

Isomer	Singly	In the presence of *l* (+) valine 1 mg./25 cc.	In the presence of *l* (+) isoleucine 1 mg./25 cc.
l (+) Isoleucine	1.7	28.7	—
d (−) Isoleucine	0.0	0.0	—
l (+) Allo isoleucine	0.0	0.0	—
d (−) Allo isoleucine	0.0	0.0	—
l (+) Valine	0.0	—	28.7
d (−) Valine	0.0	—	0.0

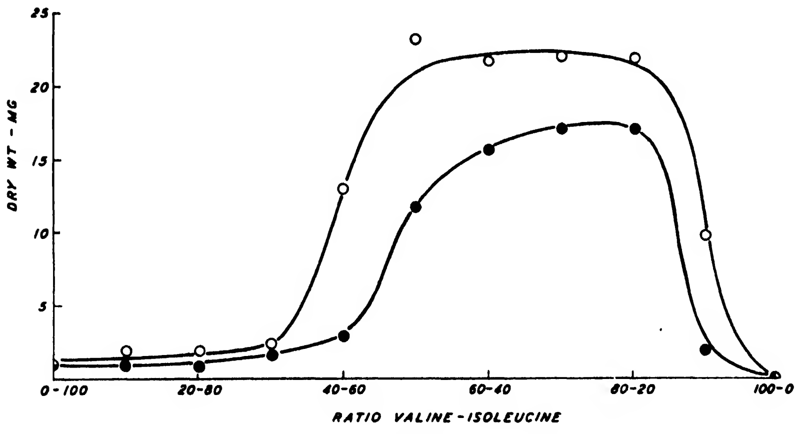


FIG. 4

The Growth Response of Mutant 16117 to Mixtures of Isoleucine and Valine of Varying Composition

Amino acids added as the racemic mixtures. Cultured 55 hours at 34°C.
○—○ Mixtures containing a total of 1.0 mg. of *l*(+) isomers per 20 ml.
●—● Mixtures containing a total of 0.5 mg. of *l*(+) isomers per 20 ml.

tested. At the present time there is little evidence concerning the actual nature of this inhibition other than the fact that it can be overcome by further addition of the mixture of active amino acids, suggesting that it may be of a competitive nature.

Different mixtures of isoleucine and valine were tested to determine whether any active mixture could account for all of the observed properties of the isolated material. On the assumption that the $l(+)$ isomers only are active, good agreement was found between the activity of a 50

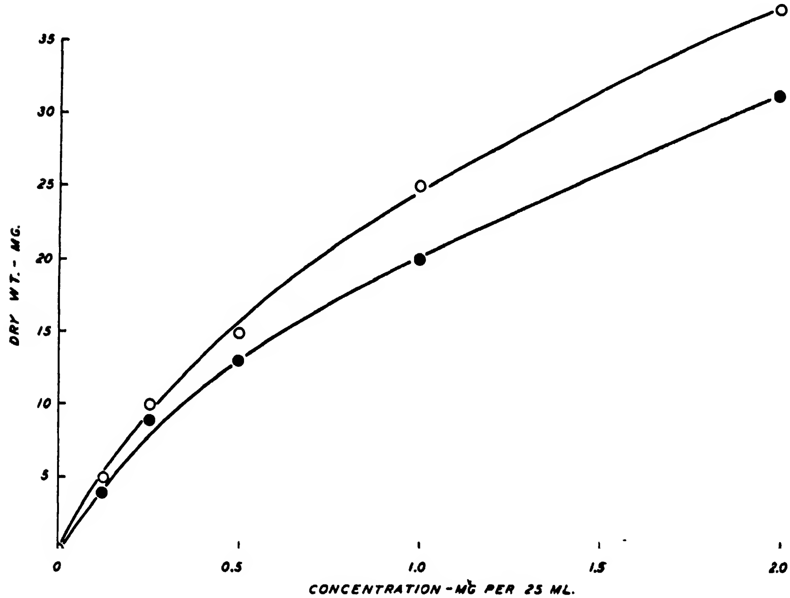


FIG. 5

A Comparison of the Activity of a Mixture Containing 70 Per Cent $l(+)$ Valine and 30 Per Cent $l(+)$ Isoleucine with the Activity of the Material Isolated from Casein as Determined by the Growth Response of Mutant 16117

Cultured 72 hours at 25°C.

○—○ Mixture of 70% $l(+)$ valine and 30% $l(+)$ isoleucine added as the racemic mixture

●—● Material isolated from casein

per cent mixture of *dl*-isoleucine and *dl*-valine, and the material isolated from casein. The six possible isomers of these two amino acids were tested, using samples prepared by resolution of the synthetic racemic mixtures. As shown in Table III, the $l(+)$ isomers only are active. The optimum mixture of these two amino acids for mutant 16117 was found to be 70–80 per cent $l(+)$ valine and 20–30 per cent $l(+)$ isoleucine,

TABLE IV

The Activity of Various Neutral Amino Acids Tested in the Presence of 3.6 mg. dl-Isoleucine and 0.4 mg. dl-Valine per 25 cc. of Medium

Cultured 50 hrs. at 34°C.

Amino acid tested	Concentration, *mg./25 cc.*	Mycelium dry weight, *mg.*
dl-Norleucine	1.0	0.0
dl-Leucine	1.0	16.4
dl-tert.-Leucine	1.0	0.0
Control		2.1

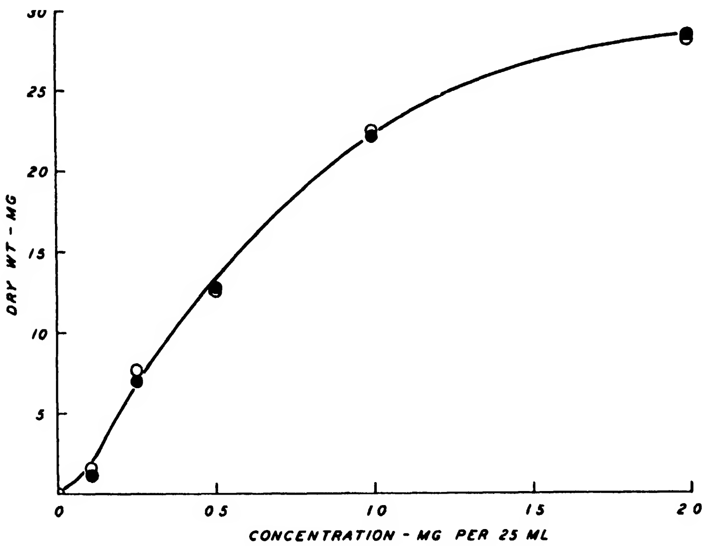


FIG. 6

A Comparison of the Activity of a Mixture Containing 60% $l(+)$ Isoleucine, 25% $l(-)$ Leucine, and 15% $l(+)$ Valine with the Activity of the Material Isolated from Casein, as Determined by the Growth Response of Strain 16117

Cultured 55 hours at 34°C.

●—● Mixture of 60% $l(+)$ isoleucine, 25% $l(-)$ leucine, and 15% $l(+)$ valine added as the resolved isomers

○—○ Material isolated from casein

as shown in Fig. 4. Such a mixture was in fact somewhat more active than the material isolated from casein (Fig. 5). The composition of a mixture containing 70 per cent valine, however, does not agree with that

indicated by the elementary analysis of the material isolated from casein, and mixtures containing 70–90 per cent $l(+)$ isoleucine and 30–10 per cent $l(+)$ valine were inactive when tested at concentrations similar to those giving good growth with the isolated product. It seemed, therefore, that a third constituent was present in the isolated product.

Tests were then carried out using a mixture of 90 per cent *dl*-isoleucine and 10 per cent *dl*-valine in the presence of other C-6 amino acids. The results of these experiments are shown in Table IV. With an insufficient supply of valine, leucine appears to be a third necessary constituent.

A mixture of 60 per cent $l(+)$ isoleucine, 25 per cent $l(-)$ leucine, and 15 per cent $l(+)$ valine has been found to have approximately the same activity as the isolated product (Fig. 6). The properties of this mixture are likewise in good agreement with those of the material isolated from casein. The calculated specific rotation of this mixture is $+30.1°$ in 20 per cent HCl as compared with the observed value of $+29.3°$. The molecular weight is about that of a C-6 neutral aliphatic amino acid, and degradation reactions would be expected to encounter the observed difficulties.

The specific requirements of this mutant strain therefore include isoleucine and valine. Under appropriate conditions leucine can reduce the amount of valine required.

Adaptation

The requirement of mutant 16117 for isoleucine and valine cannot be fulfilled by any other amino acids. The requirement can, however, be overcome by adaptation. The terms "adapted" and "adaptation" are used throughout this paper to denote a particular growth phenomenon shown by certain *Neurospora* strains when grown on incomplete media. Initiation of growth is abnormally slow, and the subsequent growth rate does not rapidly attain a constant value, but increases slowly over a period of several days. If left sufficiently long on minimal medium this mutant can become adapted to grow without the addition of isoleucine and valine. If it is grown on subminimal concentrations of isoleucine and valine it can become adapted to grow at the rate characteristic of the normal strain. It can also become adapted to grow nearly normally on isoleucine alone or valine alone. That this mutant is capable of adapting to grow on a medium containing no added isoleucine and valine does not necessarily mean that it grows without making either of these two amino acids. The more reasonable explanation would

seem to be that it is capable of making these two amino acids by some alternative mechanism, thereby circumventing the genetic block. It has been found by hydrolysis of mycelia of the normal strain and subsequent bioassay that the normal strain contains leucine, isoleucine, and valine in its proteins. It would seem, therefore, that the adapted mutant strain must have devised alternative means of making the same substances, and that the block in mutant 16117 is at a point which is rather easily circumvented by a secondary mechanism. This adds to the difficulty of interpretation as to the point of block since it is at least theoretically possible that any substance which brings about growth does so not by directly supplying the needed product of the blocked reaction but rather indirectly by accelerating adaptation. The criterion that has been used to distinguish adaptation from the normal growth response is the difference in the length of time required to attain a normal growth rate. If this criterion is valid the growth response to isoleucine and valine is normal; if, on the other hand, this criterion is not valid these two amino acids may function indirectly by accelerating adaptation.

Tests of Possible Intermediates in the Biogeneses of Isoleucine and Valine

Since the single gene change in mutant 16117 results in a requirement for both valine and isoleucine it seems likely that the biogeneses of these two amino acids pass through a common step. The available evidence suggests that in other organisms the biogenesis of leucine, isoleucine and valine proceeds by amination of the corresponding keto acids. Rose (1937) and Schoenheimer, *et al.* (1939, 1940) have shown that the rat can carry out the amination of the keto acids of these three amino acids, and is presumably unable to synthesize the characteristic branched carbon chains. The appropriate keto and hydroxy acids were therefore tested in an attempt to determine the nature of the block in strain 16117. The hydroxy acids were prepared synthetically, and the keto acids were made from racemic amino acids by the use of *d*-amino acid oxidase (see section on synthetic methods for details). In both cases the methods used left little chance of contamination by the corresponding amino acids.

The applicability of these techniques was determined by testing the hydroxy and keto acid preparations on other mutants. A leucineless mutant (strain 33757—unpublished results of D. Regnery) was found to use both the hydroxy and keto acid analogues of leucine, and a valineless

mutant (strain 33051) used the hydroxy and keto analogues of valine. In both cases response to the keto acid analogues was immediate. However, the response of the valineless mutant to the hydroxy acid analogue was delayed for 24–48 hours, and the leucineless mutant started to grow on the hydroxy acid analogue of leucine only after a small amount of leucine had been added to the medium. The subsequent rate of growth, however, appeared normal in both cases. The results indicate that the keto acid analogues are normal intermediates in the synthesis of valine and leucine by *Neurospora*, but suggest that the hydroxy acids are not. The initial lag period may represent the time necessary for the organism to adapt itself to the oxidation of the hydroxy acid to the keto acid.

TABLE V

The Activity of Leucic and α-Keto-Isocaproic Acids

Additions to the medium					
dl-Isoleucine, *mg./25 cc.*	*dl*-Valine, *mg./25 cc.*	*dl*-Leucine *mg./25 cc.*	*dl*-Caleucinate, *mg./25 cc.*	α-Keto isocaproic acid *cc. of prep./25 cc.*	Mycelium dry weight, *mg.*
Cultured 55 hrs. at 34°C.					
2.4	0.6	0.0	0.0	0.0	1.0
2.4	0.6	1.0	0.0	0.0	37.7
2.4	0.6	0.0	1.0	0.0	25.3
2.4	0.6	0.0	0.5	0.0	27.8
2.4	0.6	0.0	0.1	0.0	20.6
Cultured 72 hrs. at 25°C.					
2.4	0.6	0.0	0.0	0.0	1.1
2.4	0.6	1.0	0.0	0.0	25.3
2.4	0.6	0.0	0.0	0.1	17.6
2.4	0.6	0.0	0.0	0.01	11.8

The keto and hydroxy acid analogues of leucine can supply the leucine requirement of strain 16117 under all conditions where there is an observable requirement, that is, when valine is present but limiting (Table V). The hydroxy acid analogues of valine and isoleucine are inactive not only when tested together but also when tested singly in the presence of the other amino acid (Table VI). A mixture of the keto acids of valine and isoleucine is inactive when tested at 25° C. (Table VII). The activity of this mixture found at 34° C. is thought to be related to the increased rate of adaptation observed at this temperature. If the keto acids, however, are tested singly in the presence of the corresponding amino acids they are as active as the corresponding amino acids in both possible combinations (Table VII). This might suggest that the in-

TABLE VI

The Activity of the Hydroxy Acid Analogues of Isoleucine and Valine

Cultured 55 hrs. at 34°C.

Additions to the medium

dl-Valine, *mg./20 cc.*	*dl*-Isoleucine, *mg./20 cc.*	*dl*-Ca α-hydroxy isovalerate, *mg./20 cc.*	*dl*-Ca α-hydroxy β-methyl-*n*-valerate, *mg./20 cc.*	Mycelium dry weight, *mg.*
4.0	0.0	0.0	0.0	0.0
0.0	4.0	0.0	0.0	0.0
4.0	4.0	0.0	0.0	32.4
4.0	0.0	0.0	5.0	0.0
4.0	0.0	0.0	1.0	0.0
0.0	4.0	5.0	0.0	0.0
0.0	4.0	1.0	0.0	0.0
0.0	0.0	5.0	5.0	0.0
0.0	0.0	5.0	1.0	0.0

TABLE VII

The Activity of the Keto Acid Analogues of Isoleucine and Valine

Additions to the medium

dl-Valine, *mg./20 cc*	*dl*-Isoleucine, *mg./20 cc.*	α-Keto-isovaleric acid, *cc. of prep./20 cc.*	α-Keto-β-methyl-*n*-valeric acid, *cc. of prep./20 cc.*	Mycelium dry weight, *mg.*
Cultured 72 hrs. at 25°C.				
1.2	5.2	0.0	0.0	30.6
0.0	0.0	3.5	1.5	3.4
0.0	0.0	1.8	0.7	2.5
0.0	0.0	0.9	0.3	1.3
Cultured 55 hrs. at 34°C.				
0.0	0.0	0.1	0.5	11.9
0.0	0.0	0.1	0.1	8.4
0.0	0.0	0.1	0.01	3.7
0.0	0.0	0.5	0.1	20.0
0.0	0.0	0.01	0.1	5.3
4.0	0.0	0.0	0.0	0.0
0.0	4.0	0.0	0.0	3.0
4.0	4.0	0.0	0.0	31.0
4.0	0.0	0.0	0.5	27.8
.4.0	0.0	0.0	0.1	28.0
4.0	0.0	0.0	0.01	17.0
0.0	4.0	0.5	0.0	23.3
0.0	4.0	0.1	0.0	17.8
0.0	4.0	0.01	0.0	7.5

activity of the mixture of keto acids is due to a mutual inhibition. The activity of isoleucine is reduced 40 per cent in the presence of a mixture of α-keto isovaleric and α-keto β-methyl n-valeric acids. Valine, however, is not reduced in activity under these conditions, and the reduction in activity of isoleucine is not sufficient to account for the inactivity of a mixture of the two keto acids.

To summarize, strain 16117 can grow normally on the keto acid analogues of either valine or isoleucine only if the other amino acid is supplied. In the presence of inadequate amounts of valine, leucine is required, and can be replaced by either the keto or hydroxy acid analogue.

Discussion

If the assumption is correct that there is a one-to-one relation between the gene and primary reaction—an assumption that has been substantiated in so far as it has been possible to test it—it follows that the primary reaction blocked in mutant 16117 is common to the biosyntheses of the two amino acids valine and isoleucine. The experimental facts so far available do not permit a definite conclusion as to the nature of this common primary reaction. However, several possibilities are suggested by the data, and a brief discussion of the most likely points of block in the mutant seems justified. The correct hypothesis may be none of those mentioned, but it is also possible that with more complete information some of the inconsistencies arising from the present interpretations of the experimental data may be resolved.

One possible reaction suggested by the data is the oxidation of the hydroxy acid analogues. This interpretation is not entirely satisfactory since a mixture of the two keto acids should then be active, and since the evidence so far available indicates that the hydroxy acid analogues are not normal intermediates in the biosyntheses of these two amino acids.

A second reaction possibly involved is the amination of the keto acids. This would require the amination of the two keto acids to be catalyzed by a common enzyme, or a single common amino donor to be involved in both cases. This hypothesis seems unsatisfactory since amination of either keto acid is apparently possible in the presence of the other amino acid. The observed inactivity of either single amino acid would then suggest a block rather in the synthesis of the keto acids.

A third possible point of block is in the introduction of the β-methyl group common to both valine and isoleucine, though at present there is

no experimental support for such an interpretation. If β-methylation precedes amination the discrepancies in the two previously discussed hypotheses apply here. If β-methylation normally follows amination, the keto acids would not be normal intermediates in the syntheses of valine and isoleucine. The observed inactivity of the mixture of keto acids would then not be inconsistent with this hypothesis.

No satisfactory explanation is available for the apparent leucine requirement observed when inadequate amounts of valine are supplied. The synthesis of leucine has in no apparent way been affected in mutant 16117 since it grows normally on added isoleucine and valine. It would seem either that part of the leucine normally synthesized by this mutant may be converted to valine, or that valine may be normally transformed into leucine. In either case the requirement could be met by supplying leucine or increasing the valine supply. It is impossible to decide at this time whether a transamination or a more elaborate synthesis is involved.

Although the data presented in the present paper do not enable a clear formulation of the relationships existing between leucine, isoleucine, and valine, they do indicate a close biochemical link between these three amino acids. The data as a whole would seem to indicate that at some point in their biogeneses leucine, isoleucine, and valine may be readily interconvertible.

Genetic Data

Crosses of mutant 16117 were made with the opposite sex of an albino strain capable of growing normally on minimal medium. Cultures were grown from spores isolated at random and also from spores isolated in order from asci. Such spores were grown on complete medium and the resulting cultures tested for ability to grow on minimal medium. Of the cultures from ascospores isolated at random, 244 grew normally on minimal medium, while 273 were unable to grow on this medium. This segregation is in reasonable agreement with the 1:1 ratio expected on the basis of a single gene difference. Spores from 21 asci were dissected in order and grown in individual tubes. In all cases the observed segregations for ability to grow on minimal medium were consistent with those expected on the basis of a single gene difference. In 16 asci, segregation for the mutant gene and its normal allele occurred in the first meiotic division, whereas in 5 asci segregation was in the second division. This would indicate that the map distance between the centromere and

the mutant gene is of the order of 12 units (see Lindegren, 1941, for interpretation of ascus segregation data). Tests for mating type were made on cultures from random isolations as well as from those from isolations of ascospores in order. The resulting data indicate that inability to grow on minimal medium is inherited independently of sex and also of the sex-linked character albino.

Fourteen recovered mutant strains, chosen at random, were tested in growth tubes on crystalline material isolated from casein and found to grow at rates approximately the same as that of the original mutant strain. Two recovered strains were tested in flasks upon crystalline material obtained from casein, and on mixtures of isoleucine, leucine, and valine. No significant differences were observed between these recovered strains and the original strain. All available evidence, therefore, indicates that the characteristic difference between mutant 16117 and normal lies in a single gene.

Synthetic Methods

Preparation of tertiary Leucine (β,β-Dimethyl-α-amino-n-butyric acid)

The methods proposed by Knoop and Landmann (1914) and by Abderhalden and Rossner (1927) were carried out and found impracticable. The following method of synthesis was carried out and found satisfactory.

Diisobutylene was prepared from tertiary butyl alcohol by dehydration with H_2SO_4 (Graham, 1927). This was oxidized to dimethyl pentanone-2 using a dichromate oxidizing reagent (Butlerow, 1877), and this in turn oxidized to tertiary butyl acetic acid with hypobromite (Homeyer, 1933). α-Brom-tertiary-butyl-acetic acid was prepared and found to correspond in properties to those described by Homeyer (1933). The brom compound was then aminated using aqueous NH_4OH in a molal ratio of 1:200. The purified amino acid was found to be soluble in water and insoluble in alcohol. It can be recrystallized from 50 per cent water-alcohol mixtures without difficulty. The crystals are six-sided plates which sublime at 250°C. as described by Knoop and Landmann (1914) and by Abderhalden and Rossner (1927). The structure was confirmed by oxidation to trimethyl acetaldehyde with Chloramine T, and subsequent identification by preparation of the *p*-nitro and 2,4-dinitro phenyl hydrazones.

Preparation of α-Methyl-valine (α-Methyl-α-amino-isovaleric acid)

α-Methyl isovaleric acid was prepared from malonic ester in the manner described by Perkin (1896). The fatty acid was brominated, fractionally distilled under diminished pressure, and the brom compound aminated with aqueous NH_4OH. In view of the extreme solubility of this amino acid, the NH_4Br was removed by treatment with Ag_2O, and the amino acid finally crystallized from methanol ether solutions. Elementary analysis gave the following results:

	%C	%H	%N
Calculated for $C_6H_{13}O_2N$	54.92	10.01	10.68
Observed	54.99	10.04	10.87

Oxidation with Chloramine T yielded methyl isopropyl ketone. This amino acid is remarkable for its extreme solubility in water and alcohol. It crystallizes as short needles from methyl alcohol pyridine solutions.

Preparation of dl-α-Hydroxy-isovaleric, dl-α-Hydroxy-isocaproic and dl-α-Hydroxy-β-methyl-n-valeric acids

β,β-Methyl-ethyl-propionic acid was prepared as described by Bently (1895). Isovaleric and isocaproic acids were used as commercial preparations. The fatty acids were brominated with Br_2 and PCl_3 in the standard manner, and the bromination mixture carefully fractionated under diminished pressure. The brom acids (fractions used boiled within a degree) were hydrolyzed with aqueous $CaCO_3$ (Fischer and Zemplén, 1909) and the hydroxy acids crystallized as the calcium salts.

Preparation of α-Keto-isocaproic, α-Keto-β-methyl-n-valeric, and α-Keto-isovaleric acids

These acids were prepared by oxidation of the *dl*-amino acid using *d*-amino oxidase. The enzyme preparation was made from hog kidney according to the method of Krebs (1935). The length of time required for complete oxidation of all three amino acids under controlled conditions as determined by oxidations carried out in Warburg manometers was three hours. 300 mg. samples of the racemic mixtures of the amino acids were incubated with the enzyme at pH 8.5 for three hours. The enzyme was precipitated by boiling after adjusting the pH to about 6. The solution was then made alkaline and concentrated in vacuo to a small volume, made strongly acid, and extracted with alcohol free ether in a continuous extractor. The ether was removed from the ether extract after drying with anhydrous Na_2SO_4 and the resulting preparation neutralized and made up to a suitable concentration. This method gave little chance of contamination by amino acids, yet gave about 90 per cent recovery of the keto acids formed. The keto acid preparations gave crystalline derivatives with 2,4-dinitro phenylhydrazine and gave negative ninhydrin reactions. Controls run in an analogous manner with no substrate added gave no carbonyl compounds, and pyruvic acid was obtained when *dl*-alanine was used as substrate. Both control preparations were totally inactive when tested on normal and mutant *Neurospora* strains.

SUMMARY

1. Strain #16117 of *Neurospora crassa*, an x-ray induced mutant differentiated from normal by a single gene, grew on hydrolyzed casein and yeast-extract but not on a mixture of the known amino acids.

2. The active constituent isolated from hydrolyzed casein was identified as a mixture of 60 per cent $l(+)$ isoleucine, 25 per cent $l(-)$ leucine, and 15 per cent $l(+)$ valine.

3. Isoleucine and valine are the only amino acids actually required for growth by this mutant, which grows optimally on a mixture of 70–80

per cent $l(+)$ valine and 30–20 per cent $l(+)$ isoleucine. With an inadequate supply of valine, leucine is required.

4. Phenylalanine, norleucine, and norvaline inhibit the activity of isoleucine and valine. At concentrations similar to those of the mixtures of known amino acids originally tested the inhibition is nearly 100 per cent.

5. The hydroxy and keto acid analogues of leucine can supply the leucine requirements under all conditions of observable leucine activity. The hydroxy acid analogues of isoleucine and valine, and a mixture of the two keto acid analogues are inactive. Either keto acid analogue in the presence of the other amino acid however is active.

6. The mutant can in time adapt to grow on media containing either isoleucine or valine, or even on medium with no addition of either of these two amino acids.

7. Although it is impossible to decide what reaction is blocked in the mutant strain as compared with the normal strain, some possibilities are suggested. At some point in their biogeneses, leucine, isoleucine, and valine seem to be readily interconvertible.

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Nicotinamide Riboside

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Introduction

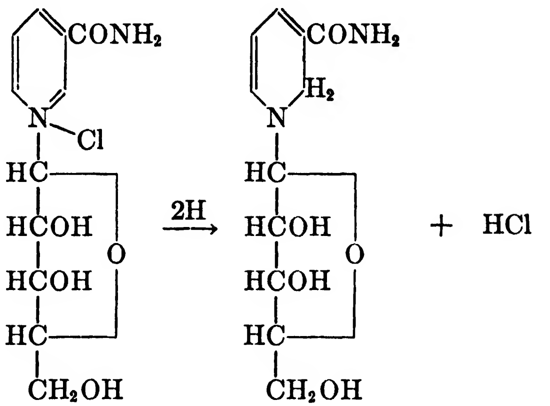
In this paper, the preparation of nicotinamide riboside from codehydrogenase I is described, and some of its chemical properties are discussed. This compound is of interest to the chemist as one of the most important structural units of the codehydrogenases, and to the biochemist, for the examination of its vitamin properties and for studies of coenzyme specificity. It is believed to be an intermediary compound in the biosynthesis of the codehydrogenases.

The preparation was accomplished by enzymatic hydrolysis of codehydrogenase I.[1] Acid or alkaline hydrolysis cannot be used, since in both instances the first result is the splitting of the linkage between the pyridinium base and the carbohydrate. The isolation from the hydrolyzate is complicated by the lability of the nucleoside. Furthermore, there are no suitable methods for its precipitation except by phosphotungstic acid. So far, the compound has not been obtained in a crystalline state, but there is evidence that the method of preparation outlined below yields the pure or almost pure product.

Nicotinamide riboside shares all characteristics with other pyridinium compounds, in which the ring nitrogen is substituted by carbohydrate or carbohydrate-like side chains. Numerous compounds of this type have been synthesized by P. Karrer and co-workers (2). It bridges the gap between these model compounds with unbiological carbohydrate groups and the coenzymes I and II. Like these compounds it is readily

[1] A preliminary report on this subject has been published earlier (1).

reduced by hydrosulfite in faintly alkaline solution to the corresponding dihydro compound:



The structure is evident from the split products, which may be obtained by acid hydrolysis: nicotinamide and pentose in a proportion of 1:1. The pentose has been identified with *d*-ribose (3).

Nicotinamide riboside does not act as a coenzyme in dehydrogenase systems, since it does not have the phosphoric acid and adenylic acid radicals necessary for combination with the protein to form dehydrogenases.

Preparation of Nicotinamide Riboside from Codehydrogenase I

Enzymatic Hydrolysis

The enzyme preparation to be used should be free from nucleosidase. The pH optimum for activity should be near the pH optimum for stability of codehydrogenase and the nucleoside. Enzyme preparations from sweet almond press cake[2] as described by Bredereck fulfill these requirements. For preparation of this enzyme the publications of Bredereck and coworkers should be consulted (4). It should be emphasized here that the decomposition of the final tannic acid precipitate by acetone should be done very carefully, since tannic acid would complicate the fractionation of the hydrolyzed cozymase.

A preliminary experiment on a small scale should be carried out to secure information concerning the activity of the enzyme preparation.

[2] Obtained from the American Almond Products Company, Glendale, California, to whom the author wishes to extend his gratitude.

The concentration of the enzyme should be such that about 50 per cent of the total phosphate of codehydrogenase I is split off after 24 to 48 hours. In the enzymatic hydrolysis the speed limiting factor seems to be the concentration of the nuclease which splits the coenzyme into mononucleotides. The subsequent liberation of phosphate from the latter is much more rapid. It was found that adenosine-5′-phosphoric acid is dephosphorylated by the enzyme preparations about five times as fast as is cozymase.

The following experimental conditions were found to be suitable: 2.0 g. of codehydrogenase I[3] are dissolved in 200 ml. of water. This solution is adjusted to pH 3.5 to 4.0 with 0.1 *N* sodium hydroxide. A 1.0 to 3.0 g. sample of enzyme preparation (depending on its activity) is extracted with 100 ml. of water at 0° C. for 1 hour. After centrifuging, the residue is extracted again with 100 ml. of water for one half hour. The combined extracts are added to the cozymase solution and the acidity is adjusted to pH 4.5. A few ml. of toluene are added as a preservative. The flask is then kept in an incubator at 30° C. Since the liberation of phosphoric acid renders the medium more acid, readjusting of the pH will be necessary after about 12 hours and again after 2 to 3 days. After 3 to 4 days a second charge of enzyme extract is added. The amount used depends on the progress of the phosphate liberation.

To secure information about the progress of the enzymatic hydrolysis the following test reactions are used.

Determination of Free Phosphate

Small samples are deproteinized by the addition of 10 per cent trichloroacetic acid. After centrifuging and suitable dilution, the phosphate determination is carried out according to Fiske and Subbarow's method. In another sample the total phosphate is determined.

Fermentation Test

Myrbäck's apozymase method was used (5). No inhibition of the fermentation by the compounds present in the enzymatic hydrolysis mixture was noticed, except in the final stage of splitting when the undiluted solution is tested for cozymase. The decrease of coenzyme activity was found to correspond to the liberation of phosphate. An example of this has been reported previously (6).

Nicotinamide Determination

Very little nicotinamide is split off during the enzymatic hydrolysis. This may be controlled by any of the numerous tests for nicotinamide, provided the conditions are such as to leave cozymase and the nucleoside intact. Therefore,

[3] Large amounts of yeast for preparation of the starting material have been donated by Anheuser-Busch, Inc., St. Louis, to whom the author wishes to express his sincerest thanks.

if the test reaction described on page 99 is to be used, no acid hydrolysis which transforms nicotinamide into nicotinic acid should be carried out, since under these conditions all nicotinamide from the nucleoside would be split off. For evaluation of the result, a standard of nicotinamide is used since the intensity of the yellow color in the cyanogen bromide reaction with nicotinamide is weaker than that obtained from nicotinic acid. For experimental conditions see page 99.

Spectrophotometric Determination

No accurate spectrophotometric determination of the total amount of pyridinium compounds is possible in these impure solutions. If such a test is desired, the reduction of cozymase and the nucleoside to the monohydro derivatives (see page 101) may be used for an approximate determination.

Complete liberation of phosphate and negative results in the fermentation test (after 4 to 7 days) indicate the end of the enzymatic hydrolysis. No traces of cozymase should be left, since it accompanies the nucleoside through most of the purification steps.

Isolation of the Nucleoside from the Hydrolyzate

Since the compound is very labile, most operations were carried out in a cold room at 0°C. For centrifugation of large volumes an International Refrigerated Centrifuge, size 3, was used. These facilities are not a prerequisite for successful preparation. In earlier work the author has succeeded in preparing the nucleoside using more moderate equipment, although the yield was smaller. The isolation takes several days and should not be prolonged unnecessarily. All precipitates are washed repeatedly.

The solution is clarified by filtration. The main portion of the phosphate and part of the protein is removed by adding 2 ml. of 1 *M* barium acetate and then dild. $Ba(OH)_2$ to pH 8. After centrifuging, the solution is readjusted with dild. H_2SO_4 to pH 4 and the barium sulfate is centrifuged off. By vacuum distillation (bath <40° C.) the volume is brought to 200 to 250 ml. Three volumes of alcohol are added, and the resulting precipitate, which contains part of the adenosine, may be worked up toward the isolation of adenosine according to the methods given in the literature. The solution is freed from alcohol by vacuum distillation to a volume of 50 ml. A 20 per cent solution of $Hg(Ac)_2$ in 2 per cent acetic acid is added and the acidity is adjusted by 0.1 *N* NaOH to pH 7. The completeness of the reaction should be assured. 10 ml. of $Hg(Ac)_2$ solution were found to be sufficient in most instances. The precipitate is centrifuged off. It contains adenosine and may be combined with the first alcohol precipitate (see above). The solution is treated with H_2S; HgS is filtered off and the filtrate concentrated by vacuum distillation to about 50 ml. Then, 1.0 *N* sulfuric acid is added to pH 2. From this solution the nucleoside is precipitated by 20 per cent phosphotungstic acid (20 to 30 ml.). The precipitate is kept for several hours at 0° C., centrifuged, and

washed twice with 5 per cent phosphotungstic acid solution, containing a small amount of sulfuric acid. It is suspended then in 50 ml. of 0.1 N H_2SO_4 and shaken with glass beads to render as fine a dispersion of the precipitate as possible. Then, 50 ml. of an amyl alcohol-ether mixture (1:1 by volume) is added. After 15 minutes of mechanical shaking the layers are separated by centrifuging. Amyl alcohol and ether are siphoned off and the precipitate is shaken up again in the dilute sulfuric acid. The amyl alcohol-ether extraction is repeated 4 to 5 times. These operations beginning with the phosphotungstic acid precipitation may be carried out in a centrifuge tube of suitable size to avoid the transfer of the mixture to and from other containers. The volume of the water layer decreases in the course of the amyl alcohol-ether extractions. By adding small amounts of 0.1 N H_2SO_4, it should be kept around 50 ml. After the final extraction, a considerable amount of a white precipitate may remain. This consists mainly of tungstic acid and may be disregarded. For the removal of traces of amyl alcohol, the solution is now extracted 3 times with a small amount of ether. To the remaining solution $Ba(OH)_2$ is added to pH 4. The precipitate is centrifuged off and the solution is concentrated to about 5 ml. by vacuum distillation. By further addition of $Ba(OH)_2$ this is adjusted to pH 6–7. After centrifuging, the volume of the solution is brought again to 5 ml.

Acetone is added drop by drop under vigorous shaking until a faint cloudiness appears. After some time at low temperature, Fraction 1 is centrifuged off. To the remaining solution, 25 ml. of acetone and 100 ml. of ether is added. After several hours, Fraction 2 is centrifuged off. Both fractions are oily. They are solidified and dried in the following manner: A mixture of dry acetone and abs. ether (1 + 4 by volume) is added. The centrifuge tube is well stoppered to keep out moisture. After 24 hours the acetone-ether mixture is separated by centrifugation and decantation. The tube is immediately connected with a vacuum pump and inserted into a water bath of about 30 to 40° to promote evaporation of adherent solvent. The oily precipitate is changed hereby into a frothy mass which slowly solidifies. The greatly increased surface facilitates further drying by another charge of acetone-ether mixture. After repeating the centrifugation and evaporation the tube is stored in an evacuated desiccator.

The yield is 50 to 100 mg. of Fraction 1; purity about 50 per cent. Fraction 2 yields 500 to 700 mg. of nucleoside, purity 60 to 80 per cent. The sulfate of the nicotinamide riboside is obtained. The degree of purity of these fractions may be determined by the spectrophotometric method, and by determination of the ratio: nicotinamide/pentose. Sometimes small amounts (<0.2%) of phosphate are found. The absence of cozymase has then to be ascertained by negative results in the fermentation test. As further impurities, small amounts of the compounds present in the original enzymatic hydrolyzate, which escaped separation, may be found. For their elimination the following method may be resorted to.

Precipitation of Impurities by Ag^+

300 mg. of Fraction 2 are dissolved in 4 ml. of H_2O and treated with 2 ml. of saturated Ag_2SO_4 solution. The precipitate is centrifuged off and to the solution dil. $Ba(OH)_2$ is added to pH 6–7. The resulting precipitate is centrifuged off. From the solution Ag^+ is removed by H_2S. After 2 hours aeration (7) Ag_2S is filtered off. In the solution SO_4^{--} is exchanged for Cl^- by addition of 0.1 *N* $BaCl_2$ solution. An excess is to be avoided. The solution is concentrated to 2–3 ml. and precipitated by acetone and ether as outlined above. The yield depends on the purity of the starting material used in this procedure. Approximately 200 mg. of a pure or almost pure preparation is obtained.

Precipitation from Methyl Alcohol Solution by Ether

This purification step may be carried out instead of the Ag^+ treatment or in addition to it. The preparation is dissolved in as little methanol as possible; a slight residue is centrifuged off, and from the solution the nucleoside is precipitated with ether. The total yield of pure nucleoside is from 25 to 40 per cent of the theoretical. So far it has not been obtained in a crystalline form. Its composition and structure, however, are beyond doubt. This is evident from the following examination of a sample obtained according to the method outlined above.

Elementary Analysis

For this, the preparation was dried in vacuo at 60°C. It was so hygroscopic that considerable moisture was attracted during the short period of manipulation between drying and inserting it into the combustion tube.[4] The result is in accordance with previous findings (1).

$C_{11}H_{15}O_5N_2Cl + \frac{1}{2}H_2O$ (MW = 299.6).

Calculated.	44.10% C, 5.37% H, 9.35% N, 11.85% Cl.
Found.	44.36% C, 5.80% H, 10.07% N, 12.11% Cl.

Determination of the Ratio: Pentose/Nicotinamide

For the determination of pentose the orcinol reaction of Bial as modified by Mejbaum (8) was used. For details see Reference (3). No hydrolysis of the nucleoside previous to the test is required.

For the determination of nicotinamide the BrCN reaction of König

[4] The author is indebted to Mr. R. H. Morris of this laboratory for this microanalysis.

as adapted by Shaw and MacDonald (9) was used with minor modifications. The nucleoside has to be hydrolyzed, since only free nicotinamide and nicotinic acid give the color reaction. The stability of the linkage between nicotinamide and pentose in the nucleoside is about the same as that in cozymase. Therefore, hydrolysis for about 45 minutes in 0.1 N HCl at 100°C. would be sufficient for complete liberation of the base. A small part of the nicotinamide, however, is hydrolyzed under these conditions to nicotinic acid. Since nicotinic acid gives about twice as strong a color as does nicotinamide under the conditions outlined below, the partial hydrolysis of the acid amide group would complicate the quantitative determination. Therefore, the hydrolysis was carried out under more rigorous conditions to ensure both the quantitative liberation of the base and its further hydrolysis to nicotinic acid. Hydrolysis at 100°C. in 1 N HCl for four hours was found satisfactory. The neutralized solution was diluted with 0.5 M NaCl solution to suitable concentration.

The composition of samples for the colorimetric test was as follows: 1.0 ml. of 1 M KH_2PO_4; 2.0 ml. of 0.5 M NaCl solution containing the nicotinic acid standard (10 to 50 gamma) or unknown; (NaCl has a profound influence on the intensity of the color in this test, therefore all samples were brought to the same NaCl concentration); 6.0 ml. BrCN solution, prepared from saturated Br_2–water and 10 per cent KCN solution; 1 ml. of 4 per cent alcoholic solution of aniline. The color reaches its maximum after 5 minutes; it is unstable. For the measurements, a Klett-Summerson photoelectric colorimeter was used.

Example: 5.52 mg. nucleoside was dissolved (without previous drying) in 5.0 ml. of 1 N HCl. For pentose determination 1.0 ml. of this was diluted with water to a volume of 50 ml. and the test carried out with 0.5, 1.0, and 1.5 ml. An average value of 8.6 γ pentose per ml. was found (standard curve with *d*-ribose). This corresponds to 2.86 gamma moles of pentose per ml. stock solution. For nicotinamide determination 2.5 ml. of the solution were hydrolyzed 4 hours in a boiling water bath. The solution was neutralized with an equal amount of 1 N NaOH. It was diluted then with 0.5 M NaCl solution to a volume of 10.0 ml. Of this 0.25, 0.5, and 0.75 ml. were used for nicotinic acid determination. An average of 88.5 gamma per ml. was found, which indicates 2.90 gamma moles per ml. stock solution. (1.10 mg. of $C_{11}H_{15}O_5N_2$ + 5 H_2O corresponds to 2.91 gamma moles). Thus, the ratio:pentose/nicotinamide was found to be 1:1.02.

Catalytic Hydrogenation

For the catalytic hydrogenation of the nucleoside essentially the experimental directions as outlined by Warburg and Christian (10) were followed, with some

modifications. Instead of platinum asbestos, a colloidal palladium solution stabilized with gum arabic (11) was used. It was saturated with a stream of hydrogen gas for one hour previous to pipetting it into the Warburg flasks. Each flask contained 1.0 ml. of colloidal palladium solution, 1.0 ml. 0.1 *M* borate buffer (pH 9.0), and in the sidearm the compound dissolved in 0.5 ml. of H_2O. The temperature was 30°C. After the hydrogen uptake was less than 1 cu.mm. per 10 minutes, the content of the sidearm was tipped. After 30 minutes, the hydrogen uptake became slow again, and after 60 to 75 minutes it was at the same rate as in the blank experiment which did not exceed 5 cu.mm. per hour. For comparison, codehydrogenase I was treated in the same way. The following values were

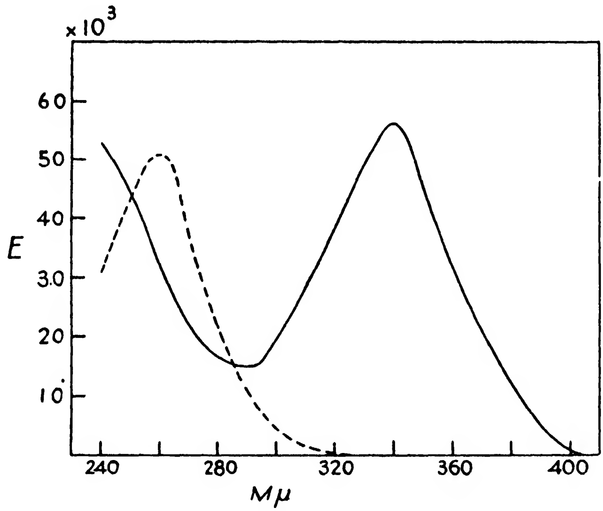


FIG. 1
Absorption Spectrum of Nicotinamide Riboside
----, oxidized form; ——, reduced form.

obtained: 0.885 γ moles codehydrogenase I: a) 58.5, b) 61.0 cu.mm. H_2 (2.68 γ moles); 1.85 γ moles nicotinamide riboside: a) 118, b) 122 cu.mm. H_2 (5.36 γ moles). It is apparent from these data that nicotinamide nucleoside yields a hexahydroderivative as do the codehydrogenases I and II.

Absorption Spectrum

Fig. 1 shows the absorption spectrum of the nucleoside in the oxidized and reduced form (dihydro derivative). The latter was prepared according to Warburg and Christian (10) using sodium hydrosulfite in bicarbonate, and stabilizing the reaction product by adding sodiumcarbonate previous to the removal of the excess of hydrosulfite by oxygen. A Hilger spectrograph (model E 498) was used. There is very

good agreement with the optical properties of the codehydrogenases I and II as well as with Karrer's pyridinium model compounds. The reduced nucleoside shows a characteristic absorption band with a maximum at 340 mμ, $E = 5.7 \times 10^3 \left[\frac{\text{liter}}{\text{mole} \times \text{cm}}\right]$. Its intensity was found to be about the same as that of the dihydrocoenzymes.[5] The oxidized compound has an absorption maximum at 260 mμ. Since this band in the absorption spectrum of the coenzymes is mainly due to their adenine content, and to a lesser degree to the pyridine base (10), it is not surprising that it is found to be much weaker in the nicotinamide riboside spectrum.

As an intermediary product of the hydrosulfite reduction, the deeply yellow monohydro compound can be observed. As has been found by Adler and coworkers (12) the monohydro derivatives of the pyridinium coenzymes can be stabilized if the reduction is carried out at pH > 10. The monohydro nicotinamide riboside can be obtained in exactly the same way. For its optical examination a Coleman Universal Spectrophotometer was used. It shows such a striking similarity with previously known monohydro compounds of this class (13) that detailed data may be omitted here.

Stability toward Chemical Reagents

Like the codehydrogenase I and II the nucleoside in the oxidized form is stable at room temperature in faintly acid medium and very labile toward alkali (splitting of the linkage between ribose and nicotinamide) (14). The contrary holds for the dihydroform: It is stable in weakly alkaline solution and very labile toward acid (addition of acid to the double bonds of the pyridine ring) (15). Hypoiodite destroys the pyridine ring (16). Bromine water and hydrogen peroxide have no influence on the oxidized compound in weakly acid solution.

Discussion

It is evident from the data reported here that all the characteristic properties of the codehydrogenases I and II, which are attributable to the nicotinamide nucleus in the quaternary linkage, are found also in the nicotinamide riboside. This compound, therefore, represents a link between the coenzymes and the synthetic model compounds of

[5] The accuracy of the method used is estimated to be ±10 per cent.

P. Karrer and coworkers. Furthermore, it has made possible the identification of the carbohydrate in the nicotinamide nucleotide moiety of the coenzymes (3). The availability of this compound has opened a field of biochemical investigation of formation and destruction of the codehydrogenases (6, 17), coenzyme specificity, and vitamin properties (18). Experimental work along these lines will be reported in further publications.

SUMMARY

1. Nicotinamide riboside can be prepared by enzymatic hydrolysis of codehydrogenase I. Its isolation from the hydrolyzate and methods of purification are described.

2. The chemical structure and various properties of the compound are discussed.

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Synthesis of *d*(−)3-Phosphoglyceric Acid and *d*(+)2-Phosphoglyceric Acid

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Introduction

The first synthesis of *d,l*-phosphoglyceric acid was described in 1928 by Neuberg, Vogt, and Weinmann (1). Metaphosphoric ethyl ester was combined with anhydrous glyceric acid and the resulting compound treated with aqueous ammonia. After elimination of inorganic phosphoric acid the neutral barium salt, $(C_3H_4O_7P)_2$ Ba_3, was precipitated. The pure substance was obtained from this compound by transformation to the easily crystallizable acid barium salt, $C_3H_5O_7PBa$. This method proved equally valuable in isolating the optically active forms (2) and is frequently employed, as it produces a characteristic, well defined, crystallized derivative of phosphoglyceric acid. The properties of this acid barium salt made it appear almost certain that the synthetic difficultly soluble crystalline product was the salt of *d,l*-3-phosphoglyceric acid. However, the possibility of simultaneous formation of isomeric 2-phosphoglyceric acid in this synthesis could not be excluded. The optically active forms of both of these isomeric compounds, as esters of *d*-glyceric acid, have been found to be universal products of the metabolism of plant and animal cells. In pure form the optically active 3-acid was first obtained by Neuberg and Kobel (2), and the 2-acid by Meyerhof and Kiessling (3). Employing the old method of Neuberg and Pollak (4), which is found (1) applicable to phosphorylation of glyceric acid, *i.e.*, treating substances containing an aliphatic hydroxyl group with $POCl_3$ in the presence of agents capable of binding free acid, Fawaz and Zeile (5) have recently prepared a phosphoric ester of unknown structure from *d,l*-glyceric acid. Another synthesis was suggested years ago by Grimbert and Bailly (6). They have shown that α as well as β-glycero-

phosphoric esters could be oxidized by bromine water without splitting off the phosphoric acid. They assumed that dihydroxyacetone phosphoric acid and phosphoglyceric acid were formed as products of the oxidation. This method, devised mainly for analytical purposes, was further developed as a method of preparation by Kiessling (7), who isolated the racemic forms of 3- and 2-phosphoglyceric acid in this manner. Both antipodes can be prepared from these substances biologically (7, 8) with the help of enzymes derived from muscle, yeasts, or lactobacilli. The unnatural *l*-form of 3-phosphoglyceric acid may be obtained equally well by bromine oxidation of *l*-α-phosphoglycerol (9).

I. S. Neuberg and R. Lewy (10) have shown that the hydrolysis of *d*(−)3-phosphoglyceric acid into H_3PO_4 and free *d*(−)glyceric acid can be carried out by means of phosphatases containing no glycolytical system. If the raw material is pure, the *d*-glyceric acid will show the maximum of optical activity. This makes *d*-glyceric acid available, as *d*(−)3-phosphoglyceric acid can be produced by yeast in any quantity (2, 11).

Starting from free *d*-glyceric acid, both the natural phosphoglyceric acids belonging to the *d*-serie may be synthesized by the above mentioned method of Neuberg, Vogt, and Weinman (1). The *d*(−)3-acid is formed as the chief product, together with a smaller amount of *d*(+) 2-acid. Both compounds are identical with the natural products and therefore completely fermentable.

It is apparent that no change in configuration is brought about by the enzymatic dephosphorylation of *d*(−)3-phosphoglyceric acid, nor does such change occur in artificial phosphorylation of optically active glyceric acid under the prevailing conditions.

The preparation of *d*(−)3- and *d*(+)2-phosphoglyceric acids described is a total synthesis. *d*-Glyceric acid may be prepared by a biochemical method[1] as well as in a purely chemical way. Neuberg and Silbermann (13) split the racemic glyceric acid into the two optical components. Furthermore, *d*-glyceric acid was prepared from the *d*-aldehydoglyceric acid, a degradation product of nitroglucose and nitrocellulose (14). Wohl (15) and H. O. L. Fischer and co-workers (16) obtained *d*-glyceric

[1] Colin and Augier (12) made the remarkable observation that *d*-gyceric acid is present as a natural glucoside in combination with *d*-mannose in algae of the genus *Polysiphonia*. The designation of "*l*-glyceric acid" given by these authors should be changed to *d*-glyceric acid in accordance with the new rational nomenclature.

acid from optically active *d*-glyceraldehyde, and Jackson and Hudson (17) established its genetic relation to various *d*-methylglycosides.

Experimental Part

The synthesis of *d,l*-phosphoglyceric acid (1) does not present any particular difficulty; the yield, however, is only moderate. Other authors, using the same method (8, 18), did not improve the yield. A few factors which will increase the yield in optically active forms will be mentioned later.

The barium salt of *d*-glyceric acid was prepared from the pure acid barium salt of *d*(−)3-phosphoglyceric acid (2). As observed by I. S. Neuberg and R. Lewy (10) takadiastase "Sankyo" is a suitable means of enzymatic splitting, whereas potato phosphatase according to Pfankuch (19) is a weaker agent. The free *d*-glyceric acid was prepared by adding the exact quantity of H_2SO_4 to the barium salt. The solution was concentrated in vacuo until a syrup was obtained which was then freed from water completely under high vacuum at from 35° to 39°C.

26.5 g of *d*-glyceric acid (0.25 mol) were mixed with 41 g (0.375 mol)[2] of metaphosphoric acid ethyl ester prepared according to Langheld (20). A clear mixture soon resulted, which subsequently became cloudy with simultaneous development of heat. For about one hour the mixture was cooled to room temperature by occasionally dipping the container in cold water. The reaction was terminated by heating two hours on a water bath while excluding moisture. Disregarding the inhomogeneity of the mixture, the contents of the flask were repeatedly shaken with glass pearls and chloroform to extract the unused metaphosphoric acid ester. The residue was dissolved in 300 cc. of water, and ammonia added until slightly acid to litmus. The liquid, after standing one hour at room temperature, was then boiled for 45 minutes to saponify the ethyl esters present. After addition of ammonia until definitely alkaline, the mixture is boiled for 15 minutes. After cooling, the inorganic phosphate was removed with a 20 per cent solution of Mg acetate until no further $MgNH_4PO_4$ was precipitated. The solution was kept in a refrigerator overnight and filtered. The presence of ammonium salts, however, proved disturbing in the subsequent precipitation of the barium salt of the phosphoglyceric acid. Ammonium salts, such as acetate, bromide, chloride, nitrate, and thiocyanate, among others, completely

[2] Formerly a larger quantity had been employed.

dissolve the difficultly soluble acid barium salt of phosphoglyceric acid, as well as the insoluble tribasic alkaline earth salts, as was previously described for the alkaline earth salts of *d*-fructose-1,6-diphosphoric acid (21).[3] Hence, $Ba(OH)_2$ was added, a slow stream of CO_2 was bubbled through the solution, which was warmed in vacuo to 35°C. and the volume kept constant by replacing water lost through evaporation. This was continued as long as NH_3 escaped from the mixture. (Freshly precipitated $BaCO_3$ as it is formed here, expels the NH_3 in the ammonium salts.) The necessary amount of $Ba(OH)_2$ and the time required to free the NH_3 in vacuo is best determined in a small aliquot. Actually, the amount required is about 110 per cent of the amount calculated from the alkaline equivalent of the magnesium acetate used and the glyceric acid and ethyl metaphosphate employed, assuming that 1 mol $C_2H_5PO_3$ will form 1 mol H_3PO_4. 4 N HBr was then carefully dropped into the mixture until a clear solution resulted. The liquid, which should amount to 250 cc., was warmed and precipitated with a solution of hot, saturated $Ba(OH)_2$. The reaction must be alkaline to phenolphthalein. 50 cc. of methanol were added, the mixture brought to a boil, filtered by suction while still hot and washed with hot water containing 10 per cent methanol. The barium precipitate included the products of phosphorylation of the *d*-glyceric acid. This precipitate was stirred in a mortar with a little water and just enough 2 N HBr to dissolve it. To this solution, which should be very slightly acid to congo red (if necessary, acidity is reduced by addition of Ba acetate), a 95 per cent solution of ethanol is added until a slight cloudiness appears. On vigorous stirring, scratching the walls of the container and storing in the refrigerator, the characteristic crystals of the acid barium salt of *d*(−)3-phosphoglyceric acid will form. Crystallization required three days. The salt was filtered by suction, washed with ice water and finally with alcohol. Yield: 18,9 g. The substance was almost pure. Two recrystallizations from water-alcohol containing a small amount of HBr will produce the typical silky lustrous crystals described for the natural product (2, 11).

$$[\alpha]_D^{18} = -13{,}27°$$

[3] Something similar may be observed with the glycerophosphates. It is known that the alkaline earth salts are more soluble in cold than in hot water. Addition of ammonium salts prevents their precipitation at boiling temperature. This is also true of the acid strontium salt of *d*-3-phosphoglyceric acid and the calcium salt of normal butyric acid. The Ca, Sr and Ba glycerophosphates are considerably more soluble in ammonium salts than in pure water. Double transformation and complex formations are responsible for these reactions.

(1,0 g of substance dissolved in 20 cc. of N HCl; c = 2,6%, relative to free phosphoglyceric acid; l = 2; α = −0,69°.)

The natural product would show $[\alpha]_D$ = 13,85° (2).

$C_3H_5O_7PBa \cdot 2H_2O$ (357,5)
Calculated: $Ba_2P_2O_7$ = 62,8; Ba = 38,4; P = 8,7%.
Found: $Ba_2P_2O_7$ = 63,0; Ba = 38,7; P = 9,0%.

The isolation of the isomeric d-2-phosphoglyceric acid presents greater difficulties. Neither concentration of the filtrate from the first crystallization of the derivative of the 3-compound in vacuo, nor the addition of a large quantity of alcohol to this filtrate with subsequent work on the resulting amorphous precipitate, yielded any results. The observation (22) that the strontium salt of d-3-phosphoglyceric acid is much less soluble in hot than in cold water proved useful. Hence, the entire filtrate from the crystallization of the raw acid barium $d(-)$3-phosphoglycerate was freed from barium by addition of Na_2SO_4, neutralized with NaOH, concentrated to 35 cc., and mixed with sufficient hot saturated solution of $Sr(OH)_2 \cdot 8H_2O$ to give no further precipitate. This precipitate, after centrifugation, was washed, also by centrifugation, first with ice water and then with methanol and finally dried in vacuo. The strontium salt was stirred in a mortar with 40 cc. of water, and a solution of 0.5 M $HClO_4$ added drop by drop until the salt dissolved. On addition of twice the volume of alcohol a permanent cloudiness resulted and a small quantity of the acid strontium salt of d-3-phosphoglyceric acid was obtained as a microcrystalline precipitate on boiling. This was filtered while hot. To the filtrate a 15 per cent solution of basic lead acetate, $(CH_3 \cdot COO)_2Pb \cdot Pb(OH)_2$, was added until alkaline to litmus. Subsequently, a 30 per cent solution of normal lead acetate was added until no further precipitate was formed. The precipitate was centrifuged, washed three times with a solution of 10 per cent alcohol and carefully ground with H_2O. This aqueous suspension was decomposed with H_2S and the solution of free phosphoglyceric acid was freed from H_2S by CO_2. After elimination of the dissolved carbonic acid, the solution was treated with a hot saturated solution of $Ba(OH)_2$ in a medium alkaline to phenolphthalein until complete precipitation occurred. The neutral barium salt was changed to the acid salt by HBr and then precipitated with alcohol. The precipitate, which was not distinctly crystalline, was filtered by suction, washed with dilute alcohol and dried in the desiccator. The salt was almost completely dissolved in a large quantity of boiling water. The filtered solution

was then concentrated on a water bath until the first crystals appeared around the edges. These crystals still included some salt of the *d*-3-acid and were filtered. The filtrate was then concentrated until a crystal fuzz formed at the edge of the container. When slowly cooled and allowed to stand, a salt crystallized which consisted almost exclusively of the desired isomeric compound, but still included some amorphous particles. Regular recrystallization was now possible. The acid barium salt of *d*-2-phosphoglyceric acid was finally obtained by three recrystallizations, each time disregarding the first crystals obtained. The crystals consisted of thin plates.

Optical activity of the substance dried in vacuo:

$$[\alpha]_D^{22} = +23,2^\circ.$$

(0,481 g substance dissolved in N HCl and brought up to 20 cc. Relative to free phosphoglyceric acid is c = 1.25, l = 2, $\alpha = +0,58^\circ$). $[\alpha]_D^{20} = +23,55^\circ$ to $24,5^\circ$ has been indicated for the natural compound.

Indications for the water of crystallization content of the natural compound vary from 1,5 to 3 moles of H_2O. Therefore, the water-free silver salt was prepared. 0,3 g of the acid barium salt were transformed with the exact amount of K_2SO_4. The filtrate from the $BaSO_4$ was exactly neutralized with KOH and concentrated to 2 cc. The white silver salt was precipitated with concentrated $AgNO_3$ from the clear liquid. This was then filtered by suction, washed with ice water, and the substance, while still wet, dissolved in the necessary quantity of NH_4OH. This solution was neutralized with 40 per cent lactic acid in alcohol. After being kept in the refrigerator, the silver salt crystallized from the solution which had been filtered. The salt was dried in vacuo over P_2O_5 and paraffin, after having been filtered by suction and washed first with dilute alcohol and then with absolute alcohol and ether.

$C_3H_4O_7PAg_3$ (506,3).
Calculated: $Ag_3PO_4 = 82,7$; $Ag = 63,9$; $P = 6,1\%$.
Found: $Ag_3PO_4 = 82,3$; $Ag = 63,4$; $P = 5,9\%$.

Yield of *d*-2-phosphoglyceric salt amounted to 1,8 g.

Altogether, 20,7 g. of the acid barium salts of phosphorylated *d*-glyceric acid were isolated, amounting to 23 per cent of the theoretical quantity. The yield of products determined is about 6 per cent higher than that found previously obtained on similar treatment of *d,l*-glyceric

acid. It is reasonably certain that the racemic phosphoglyceric acid previously obtained by this method consisted of the 3-acid; this is certainly true of the difficultly soluble crystalline acid barium salt. (Kiessling's indication (7) that the acid barium salts of the *d,l*-phosphoglyceric acids do not crystallize, is not valid, at least not for the salt of the *d,l*-3-acid. This salt crystallizes (1, 23) in thin rods which combine to form clusters.)

The slight increase of yield results from three conditions: a) avoidance of a considerable surplus of ethyl metaphosphate during phosphorylation; b) elimination of ammonium salts which interfere with the isolation of the acid strontium or barium salts by causing solution of all alkaline earth salts of the phosphoglyceric acids; c) improved method of isolation.

Finally, both the *d*-phosphoglyceric acids have been fermented by active zymase extract in the manner previously described for the fermentation of racemic 3-phosphoglyceric acid (24) and for the natural *d*-component (2). The procedure used in the fermentation of the natural *d*-2-phosphoglyceric acid is quite similar (3). Both of the isomeric compounds ferment completely. In this way, the identity of the synthetic and the natural product is further proved by biological experiment.

Summary

d(−)Glyceric acid which may be prepared by purely chemical or biological methods, is phosphorylated by metaphosphoric ethyl ester without change of configuration. This synthesis yields preponderantly *d*(−)3-phosphoglyceric acid, and some *d*(+)2-phosphoglyceric acid, in a ratio of about 10:1.

The synthetic products are identical with the natural ones as to structure, form of crystals, optical activity, and fermentability.

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Effect of the Composition of the Diet on the Vitamin Content of Rat Tissues[1]

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Introduction

Pork, veal, and lamb carcasses have been found to vary markedly in their vitamin content, McIntire, *et al.* (1), Schweigert, *et al.* (2), McIntire, *et al.* (3) and Rice, *et al.* (4). Studies have been carried out by several workers to show the effect of diet on the vitamin content of rat tissues. Sarett and Perlzweig (5) reported that the thiamine content of both liver and muscle tissue varied with the thiamine intake, while the intake level of nicotinic acid or riboflavin did not affect the concentration of these vitamins in the tissues. Fraser, *et al.* (6), and Axelrod, *et al.* (7) found that the riboflavin content of the tissues was low, when rats were fed a riboflavin deficient ration.

Sure and Ford (8) who used a paired feeding technique, showed the effect of thiamine and riboflavin deficiencies on the thiamine and riboflavin content of several rat tissues. A 20 μg. per day intake of riboflavin and thiamine resulted in a considerable increase in the vitamin content of the tissues as compared to the pathological controls. They concluded that in a thiamine deficiency, riboflavin metabolism was disturbed, as evidenced by lower riboflavin content of the tissues when rats had been on a thiamine deficient diet as compared to rats which received 20 μg. of thiamine per day.

Schultz, *et al.* (9) have shown that the level of thiamine in the tissues can be correlated with the level of thiamine fed, however, when more than 65 μg. of thiamine per day were fed, no increase in body concentration was observed.

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The effect of the level of carbohydrate, protein, and fat as well as the level of vitamin intake on the vitamin content of the tissues of rats might indicate the type of diet which was most favorable to vitamin saturation of the tissues or changes in retention of one vitamin concurrently with a low intake of another vitamin. Thiamine, riboflavin, and nicotinic acid, and proximate analyses reported in this paper were carried out on muscle and liver from rats which received low and high levels of thiamine and riboflavin, carbohydrate, protein, and fat.

Experimental

Weanling, male rats (weight 35–45 g.) were obtained from Sprague-Dawley. Eight rats were placed in each of three groups, one group received a high carbohydrate diet, one a high protein diet, and one a high fat diet (Table I). The rations were mixed at weekly intervals and fed *ad libitum*. The rations were kept in a refrigerator when not in use.

TABLE I

Composition of Diets

Constituents	High carbohydrate diet	High protein diet	High fat diet
Sucrose	73%	41%	12%
Casein	18%	50%	29%
Salts IV	4%	4%	6%
Corn oil	5%	5%	5%
Purified lard	0	0	48%
Nicotinic acid	25 μg./day	25 μg./day	25 μg./day
Pantothenic acid	200 μg./day	200 μg./day	200 μg./day
Pyridoxin	25 μg./day	25 μg./day	25 μg./day
Choline	10 mg./day	10 mg./day	10 mg./day
Thiamine and riboflavin (see text)			

The vitamins[2] were supplemented by dropper daily. Two rats in each of the three groups mentioned above received low thiamine supplement (8 μg. per day); two a high thiamine supplement (50 μg. per day); two a low riboflavin supplement (8 μg. per day); and two a high riboflavin supplement (60 μg. per day). The low and high thiamine groups received 30 μg. of riboflavin per day, and the low and high riboflavin groups received 25 μg. of thiamine per day. The vitamin supplements

[2] Supplied by Merck and Company.

were stored in dark colored bottles in the refrigerator. One drop of haliver oil[3] was given weekly.

The rats were kept on experiment for seven weeks. Growth records were kept and the average gain per week for each group is shown in Table II.

TABLE II

Rat Growth Rates

(In grams per week)

Diet	High carbohydrate	High protein	High fat
8 μg. Thiamine/day	20	22	23
50 μg. Thiamine/day	29	26	28
8 μg. Riboflavin/day	16	24	17
60 μg. Riboflavin/day	26	27	27

TABLE III

Proximate Analysis of Rat Tissues

(All values in per cent)

Diet	Muscle tissue			Liver	
	Water	Fat	Residual solids	Water	Dry matter
High carbohydrate—low thiamine	67.2	10.3	22.5	67.0	33.0
High protein—low thiamine	70.5	6.7	22.8	67.3	32.7
High fat—low thiamine	71.3	6.8	21.9	59.2	40.8
High carbohydrate—high thiamine	68.9	8.8	22.3	68.3	31.7
High protein—high thiamine	69.2	8.5	22.3	67.2	32.8
High fat—high thiamine	70.2	7.1	22.7	59.4	40.6
High carbohydrate—low riboflavin	70.8	5.8	23.4	68.6	31.4
High protein—low riboflavin	71.2	5.7	23.1		
High fat—low riboflavin	70.2	6.9	22.9	65.4	34.6
High carbohydrate—high riboflavin	69.8	6.6	23.6	67.6	32.4
High protein—high riboflavin	69.4	7.1	23.5	68.0	32.0
High fat—high riboflavin	70.5	6.9	22.6	56.7	43.3

Preparation of Tissues for Analysis

The food and supplements were withheld from the rats for 24 hours, and the rats were then sacrificed by severing the head and allowing the

[3] Supplied by Abbott Laboratories.

TABLE IV

Vitamin Content of Tissues from Rats on Diets Varying in Carbohydrate, Protein, Fat, and Vitamin Content

(All values in μg./g.)

	Thiamine				Riboflavin				Nicotinic acid			
	Muscle		Liver		Muscle		Liver		Muscle		Liver	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
High carbohydrate—low thiamine	.30	.91	1.02	3.10	2.58	7.8	31.6	96	40	122	110	337
High protein—low thiamine	.34	1.16	.98	3.00	2.50	8.5	33.7	103	40	135	81	246
High fat—low thiamine	.28	.96	.53	1.30	2.73	9.5	26.1	64	35	122	79	193
High carbohydrate—high thiamine	1.21	3.90	4.82	15.2	2.78	8.9	30.4	96	30	97	106	335
High protein—high thiamine	.94	3.04	3.78	11.5	2.33	7.6	33.1	101	27	88	92	279
High fat—high thiamine	.60	2.00	2.68	6.6	2.58	8.7	23.6	58	38	127	115	283
High carbohydrate—low riboflavin	1.02	3.48	4.55	14.5	1.83	6.3	18.8	60	40	137	92	293
High protein—low riboflavin	.61	2.13		7.6	1.95	6.8		74	40	138		320
High fat—low riboflavin	1.05	3.55	3.46	10.0	2.13	7.2	17.7	51	50	168	123	356
High carbohydrate—high riboflavin	.76	2.50	2.46	7.6	2.62	8.7	35.0	108	44	145	111	342
High protein—high riboflavin	.65	2.11	2.24	7.0	2.52	8.3	33.6	105	40	131	95	296
High fat—high riboflavin	.73	2.48	1.52	3.5	3.80	12.9	39.0	90	44	148	99	228

animals to bleed thoroughly. The muscle tissue was removed from the front and rear legs, minced well, and the tissues from both rats in each group were thoroughly mixed together. Portions were then weighed immediately for proximate and vitamin analysis.

The livers from both rats were removed and the composite weighed immediately in tared evaporating dishes. The livers and tissues were dried in a 50° C. drying room for 48 hours. The vitamin content of the livers and the thiamine content of the muscle tissue were determined on the dry basis and calculated to the fresh basis. The riboflavin and nicotinic acid contents of the muscle tissue were determined on the fresh basis and calculated to the dry basis from the proximate analysis data.

The moisture and fat determinations were carried out by the method described by McIntire, *et al.* (1). The proximate analyses results on the liver and tissue are shown in Table III. Fat determinations were not carried out on the livers. Part of one sample of liver was lost during the drying process, consequently the vitamin content of the fresh liver could not be calculated.

The method of Hennessy (10) with modifications of McIntire, *et al.* (1) was used for the thiamine determinations. The nicotinic acid content was determined by the method of Snell and Wright (11) after extraction with 1 *N* NaOH. The riboflavin determinations were made by the method of Snell and Strong (12) with modifications reported by McIntire, Schweigert, and Elvehjem (3).

The riboflavin content of the livers was checked with the fluorometric method, and the results were in good agreement. The fluorometric method used was essentially that of Conner and Straub (13). The riboflavin figures reported on the liver analyses are an average of the results obtained by the two methods. The results of the vitamin analyses in µg. per g. of fresh and dry tissues are given in Table IV.

Discussion

The average gain in weight per week observed over the seven week growth period on the optimum diets (high thiamine and high riboflavin groups) was 27 g. per week, which is comparable to growth rates observed on complete synthetic diets by other workers. The level of carbohydrate, protein, or fat did not affect the rate of growth. An increase of approximately eight grams gain per week was noted on the high protein diet as compared to high fat or high carbohydrate diets for rats which received 8 µg. of riboflavin per day. This may be due to small

amounts of riboflavin contributed by the high intake of protein (casein) which may have contained some riboflavin, or due to a lower requirement of riboflavin when a higher level of protein was fed.

The moisture and fat contents of the muscle tissues do not vary markedly, regardless of the level of vitamin, carbohydrate, protein, or fat intake. The per cent of moisture and dry matter varied somewhat in the livers, however (Table III).

The thiamine content of both muscle and liver from rats which received the low thiamine supplements was much lower than the controls which is in agreement with the results obtained by Schultz, *et al.* (9) and Sarett and Perlzweig (5) on rat tissues. The same results have been obtained on the thiamine content of human tissues by Ferrebee, *et al.* (14) who found that the heart, muscle, liver, and kidney cortex all contained less thiamine per gram of tissue from patients with poor nutrition as compared to patients with a higher thiamine intake. They found the tissue thiamine concentrations could be elevated somewhat with an increased thiamine intake.

The thiamine content per gram of fresh rat muscle tissue on an intake of 8 μg. of thiamine per day averaged 0.30 μg. per g. as compared to 0.84 μg. per g. from rats which received 25 and 50 μg. of thiamine per day. The tissue thiamine concentrations are in good agreement with the results reported by Schultz, *et al.* (9), Sure and Ford (8), and Mitchell and Isbell (15), but are somewhat lower than the values reported by Sarett and Perlzweig (5).

Similar results were obtained on the thiamine content of the livers as shown in Table III. The thiamine content of fresh livers on the low thiamine supplement averaged 0.87 μg. per g. as compared to an average of 3.20 μg. per g. on the high thiamine supplement. These results are comparable to the results obtained by Schultz, *et al.* (9) for a 25 μg. per day thiamine intake, but they observed an increase in liver thiamine concentration on higher levels of thiamine supplementation. Sure and Ford (8) and Mitchell and Isbell (15) observed somewhat higher thiamine values on rat livers. The concentration of thiamine in the liver was somewhat lower for rats which received the high fat diets as compared to high protein or high carbohydrate on the same level of thiamine intake.

The riboflavin content of the muscle tissue was somewhat lower in both liver and muscle from rats which received 8 μg. of riboflavin per day as compared to rats which received 30 and 60 μg. of riboflavin per

day. The highest value on the muscle tissue was observed on the high fat, high riboflavin group, 3.80 μg. per g. The average value for the 30 and 60 μg. riboflavin intake per day was 2.72 μg. per g. of fresh muscle as compared to 1.97 μg. per g. for the 8 μg. riboflavin intake. Sarett and Perlzweig (5) did not obtain any difference in riboflavin content of the tissues when the level of riboflavin intake was increased. The riboflavin content of the livers on the 30 and 60 μg. per day intake averaged 31.8 μg. per g. which agrees well with the values reported by Axelrod, *et al.* (7), Mitchell and Isbell (15), Sarett and Perlzweig (5), and Sure and Ford (8).

The nicotinic acid values varied from 27–50 μg. per g. of fresh muscle tissue, and 79–123 μg. per g. of fresh liver. All animals received 25 μg. of nicotinic acid supplement per day, and since nicotinic acid has been shown to be synthesized by the rat [Dann (16) and Huff and Perlzweig (17)] it would seem likely that the nicotinic acid content of the rat tissues would be approximately the same. The average nicotinic acid content of fresh muscle tissue was 39 μg. per g.; and for fresh liver, 104 μg. per g. These values agree very well with the results reported by Sarett and Perlzweig (5), but are somewhat lower than the value reported by Mitchell and Isbell (15).

The results of these experiments show that the thiamine and to a lesser extent, riboflavin content of both rat muscle and liver vary with the specific vitamin intake. High carbohydrate, protein, or fat diets under the conditions of this experiment did not cause any appreciable differences in the vitamin content, although some variations were observed, particularly on the high fat diets. It appears therefore, that variation between rats in the vitamin content of the tissues can be correlated to a greater extent with the level of vitamin intake (thiamine and riboflavin in this experiment) than with the level of carbohydrate, protein, or fat in the diet. These observations were made on a subminimal level of thiamine and riboflavin intake. A vitamin deficiency was not produced, the only gross difference was a retardation in growth on low thiamine or riboflavin intake in comparison to growth on higher levels of thiamine or riboflavin intake.

Summary

1. Thiamine, riboflavin, and nicotinic acid contents of muscle and liver have been determined from rats which received low and high levels of thiamine, riboflavin, carbohydrate, protein, and fat.

2. The thiamine content of rat muscle and liver was 3–4 times higher when 25–50 μg. of thiamine were fed than an 8 μg. per day intake.

The riboflavin content was 40 per cent higher in the muscle tissues when 30 and 60 μg. of riboflavin were fed than an 8 μg. per day intake.

3. High carbohydrate, protein, or fat did not appreciably affect the vitamin content of the tissues when the same level of vitamin was fed, although there was some variation between groups.

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Influence of Some Environmental Factors upon the Production of Riboflavin by a Yeast

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Introduction

The production of extraordinary amounts of riboflavin (vitamin B_2) by the yeast *Candida guilliermondia* cultivated in chemically defined media offers a convenient means of studying the influence of various factors upon bio-synthesis of the vitamin. A brief description of certain phases of these investigations having been presented elsewhere (1), the present paper gives an account of observations made on the production of riboflavin in relation to the amount of inoculum, duration of the growth period, and the carbon and organic nitrogen compounds supplied in the medium. The influence of mechanical agitation is also discussed briefly.

Experimental

Material and Methods

The yeast known as strain No. 488, isolated originally from sour milk by Dr. L. J. Wickerham, was carried on nutrient agar slants to be used for inoculating the various media. The composition of each liter of the basal medium employed is as follows: KH_2PO_4, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $CaCl_2 \cdot 2H_2O$, 0.3 g.; $(NH_4)_2SO_4$, 2.0 g.; KI, 0.1 mg.; dextrose (C.P.), 20 g.; asparagine, 2.0 g.; biotin methyl ester, 1.0 μg. Trace elements were added in p.p.m. as follows: B, 0.01; Mn, 0.01; Zn, 0.07; Cu, 0.01; Mo, 0.01; and Fe, 0.05. In different experiments the source of carbon or nitrogen was varied in ways mentioned in the discussion of individual experiments. Distilled water was used in making the medium and the reaction of the solution was adjusted to pH 5.0. At the end of the growing period of a week, the reaction of the fermented liquor was about pH 3.8. Twenty-five ml. of medium were dispensed into each 125 ml. Erlenmeyer flask used for the cultures, and sterilization was accomplished by autoclaving at 120°C. for 15 minutes. After cooling, the media in the flasks were inoculated by adding, aseptically, a small measured amount of yeast suspension.

After incubating at 30°C., usually for a period of six days, growth of the yeast cultures was measured turbidimetrically, appropriate corrections being made for the colored solutions. The cultures were then autoclaved, and the yeast was removed from the fermented liquor by filtration through supercel. Riboflavin contained in the filtered fluid was determined photometrically by absorption of blue light; microbiological assays on some of the materials with *Lactobacillus casei* gave good agreement with the photometric determinations. In previous studies it was found that nearly all the vitamin B_2 of the cultures is present in the fermented liquor; hence the determinations may be regarded as approximate values for the vitamin B_2 content of the total cultures produced under the specified conditions.

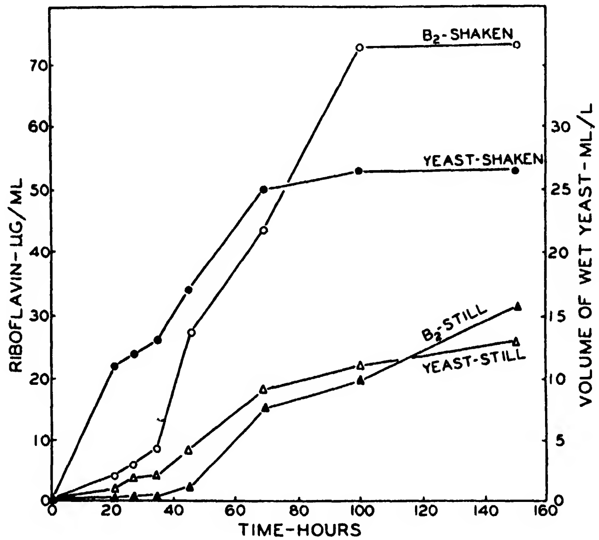

FIG. 1

Growth of Yeast and Production of Riboflavin in Still and Shaken Cultures after Different Periods of Incubation

Mechanical Agitation

For the purpose of determining the course of growth and riboflavin production during the period of incubation, two series of cultures were set up under identical conditions except that the flasks in one series were agitated continuously at slow speed on a Cenco-Meinzer mechanical shaker while the flasks in the other series were kept still. At different intervals of time three cultures were taken for measurements of yeast growth and vitamin B_2 content of the fermented liquor. The resulting data are plotted in Fig. 1. The increase in growth and vitamin

production in the shaken cultures over the still cultures is very striking. During the early part of the incubation period in both series, growth of the yeast took place rapidly and riboflavin appeared in the medium. The maximum yields of riboflavin in shaken cultures of this experiment reached about 75 μg./ml. in fermented liquor containing 27 ml. of wet yeast (9 g. dry) per liter after four days. In other experiments where the conditions were slightly modified, crystalline riboflavin accumulated at the bottom of the flasks, indicating supersaturation of the fermented liquor. In such cultures the masses of yeast cells which were filtered off appeared orange red in color. The rest of the experiments reported in this paper were performed with still cultures.

One of the most striking features of this yeast's fermentation is the accumulation of riboflavin in the medium surrounding the organisms. If the vitamin B_2 molecule is synthesized inside the yeast cells, then apparently only a small fraction of the total amount is bound to protein carriers. The explanation for apparent loss of the bulk of the riboflavin through the cellular membranes is not clear, but the phenomenon probably is related to inability of the species to elaborate an efficient phosphate-protein binding system. It seems possible that continuous diffusion of riboflavin from the yeast cells may operate through the law of mass action, to permit further synthesis of the vitamin product.

Amount of Inoculum

The strength of the inoculum is known to influence the synthetic activities of microorganisms grown under special conditions, and it seemed desirable to determine the effect of quantity of inoculum of this yeast upon growth and the production of riboflavin. Yeast grown either on nutrient agar or in liquid medium was suspended in basal medium to produce a suitable inoculum which could be added in varying amounts to the culture solutions. Cultures were set up in triplicate with seven different amounts of inoculum. After a period of six days, growth and vitamin B_2 were determined in the cultures.

The results of a typical experiment are shown in Fig. 2. Growth of the yeast, as indicated by the final crop at the end of the period, was not influenced greatly by the amount of inoculum in the range from 0.1 to 30.0 mg. of yeast added to each 25 ml. of culture medium. The yield of riboflavin was not influenced appreciably within the range of 0.1 to 3.0 mg. of yeast in the inoculum, but above this range a marked decrease in production of vitamin B_2 occurred. In a series in which the inoculum

was taken directly from a culture grown in basal solution, a similar set of results was obtained. A satisfactory explanation for these observations is not known, but it seems probable that certain compounds present in large amounts of inocula may tend to inhibit the production of riboflavin. It may be pointed out that hydrolyzed casein, urea, and certain carbohydrates, have been found to inhibit production of riboflavin. Hence it is not surprising that materials present in yeast should be found to depress the formation of the vitamin.

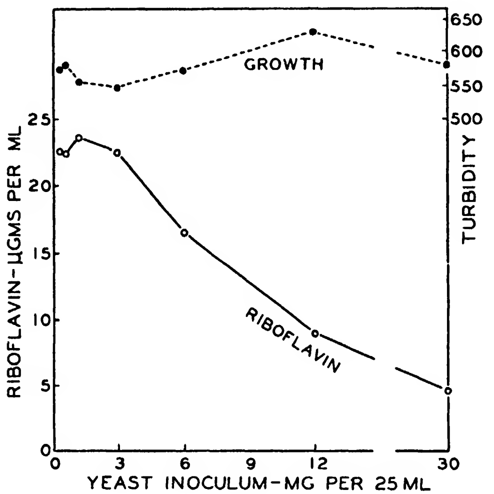

Fig. 2

Growth of the Yeast

Is not greatly affected by wide differences in strength of the inoculum, but the production of riboflavin is diminished by amounts greater than about 3 mg. of moist yeast per 25 ml. of medium

Source of Carbon

Earlier work indicated that a suitable source of carbon is important for growth of the yeast and production of riboflavin. The results of further experiments are described here to indicate the special influence of various carbon compounds supplied in the medium. Different compounds were added singly in the amount of 20 g./l. of medium, and the results observed at the end of six days.

The resulting data for 13 substances are shown in Fig. 3. It is obvious that not all compounds are equally effective for growth. Lactose and

glycerine are relatively inefficient, while other materials, not shown in the graph, such as glycogen, corn starch, ethyl cellulose, and dextrin do not support growth. Furthermore, in the group of carbohydrates which produce growth there is considerable variability with regard to their utilization for synthesis of riboflavin. Although excellent multiplication of the yeast occurred in media supplied with single additions of galactose, inulin, mannitol, arabinose, xylose, sorbose, and maltose, the yields of vitamin B_2 with these carbohydrates were relatively poor. Good yields

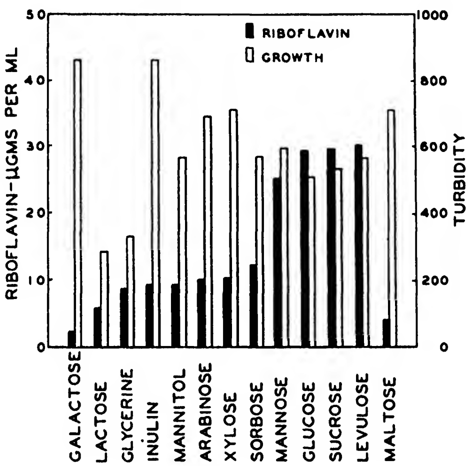

FIG. 3

Growth of Yeast and Production of Riboflavin

In relation to different sources of carbon supplied at the rate of 20 g./l. of medium. Multiplication of yeast in carbohydrate solutions is not always accompanied by high yields of vitamin B_2 in the fermented liquor

of the vitamin and satisfactory growth were observed in media containing mannose, dextrose, levulose, or sucrose.

In view of the poor production of riboflavin in media containing either galactose or the disaccharide lactose which yields some galactose upon hydrolysis, it seemed desirable to determine the extent of the inhibitory action of galactose when supplied in varying amounts to media containing dextrose. Three series of media were accordingly set up with 5, 20, and 30 g. of dextrose/liter. Galactose (Pfanstiehl, C.P.) was supplied to triplicate flasks in each series at the following levels in g./l.: 0, 0.5, 1.0, 5.0, 10.0, and 20.0. The resulting data for growth and vitamin B_2 are

plotted in Fig. 4. Multiplication of the yeast was not inhibited with increasing doses of galactose, but the production of riboflavin was limited greatly by increasing doses of this sugar up to about 5.0 g./liter. With increasing dosages above the latter level, further inhibition was much less obvious. It is interesting to note that the inhibitory influence of a given dose of galactose upon vitamin B_2 formation is about the same at all three levels of dextrose supplied in the medium.

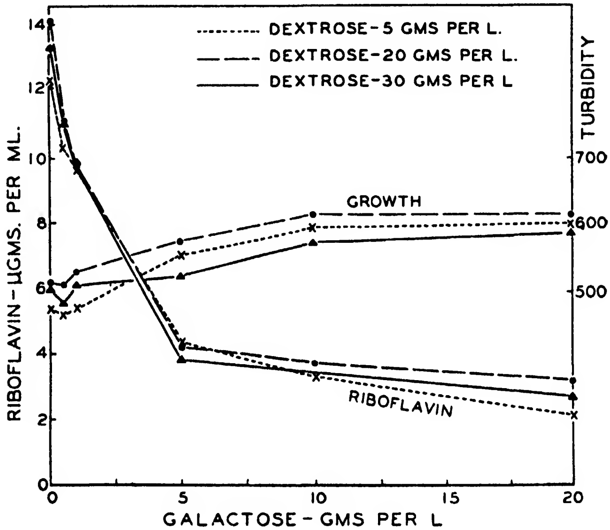

Fig. 4

Growth of Yeast and Production of Riboflavin in Dextrose Culture Media Containing Varied Amounts of Galactose

While growth is enhanced somewhat by galactose, the yields of vitamin B_2 are markedly reduced by the presence of this sugar in the medium

Source of Nitrogen

In an earlier report (1) it was shown that the source of nitrogen is important for the production of riboflavin. An experiment was designed to test the effectiveness of asparagine and various amino acids supplied in addition to $(NH_4)_2SO_4$ in the medium. Ammonium sulfate was present in the amount of 2.0 g./l. in all the media. Asparagine was supplied at the rate of 2.0 g./l. and various amino acids in the amount of 0.8 g./l. were added singly as supplements to the asparagine. As shown in Table I, the addition of asparagine and certain other amino acids mark-

edly stimulates the production of riboflavin in the fermented liquor. Small amounts of urea (0.5 g./l.) supplementing ammonium sulfate in the basal medium likewise enhance the production of the vitamin, but larger amounts (2–4 g./l.) are inhibitory.

Variations in the total amount of organic nitrogenous compounds supplied in the culture liquid greatly influences the yield of riboflavin. An experiment was performed in triplicate for the purpose of discovering

TABLE I

Production of Riboflavin in Culture Media Supplied with Various Nitrogen-Containing Compounds

Growth period 6 days, temperature 30°C. The nitrogen compounds were employed as follows: $(NH_4)_2SO_4$, 2.0 g./l.; asparagine, 2.0 g./l.: amino acids, 0.8 g./l.

Nitrogen compounds supplied in medium, *g./l.*	Riboflavin in fermented liquor, *μg./ml.*
$(NH_4)_2SO_4$	12.6
" + asparagine	18.0
" + " + *l*-tryptophan	17.4
" + " + *l*-histidine	17.7
" + " + *dl*-lysine	18.4
" + " + *l*-tyrosine	19.4
" + " + *dl*-α-alanine	25.3
" + " + *dl*-glutamic acid	27.8
" + " + *l*-cystine	28.1
" + " + *dl*-serine	31.8
" + " + *l*-proline	32.1
" + " + *dl*-phenylalanine	32.2
" + " + *l*-hydroxyproline	32.6
" + " + *dl*-aspartic acid	35.5
" + " + *d*-arginine	37.5
" + " + *dl*-leucine	40.3
" + " + *dl*-valine	43.4
" + " + glycine	56.0
" + " + *dl*-methionine	60.0

the quantitative relationship between riboflavin synthesis and increasing supplies of glycine and asparagine added as supplements to the ammonium sulfate in the basal medium. The amount of these compounds supplied singly or together was varied over the range from 0.1 to 8.0 g./liter. The results of determinations of the vitamin B_2 produced in response to the various treatments are shown in Fig. 5. The data indicate that asparagine produces yields of the vitamin about twice as

great as those obtained with glycine. The presence of both glycine and asparagine in equal proportions also produces good yields. It appears that about 2 g./l. of these compounds result in nearly maximum production of the vitamin for the particular medium employed. Although the best combination studied so far was asparagine plus methionine, the latter would appear to be too expensive for use in commercial production of the vitamin. By shaking the cultures in further experiments the

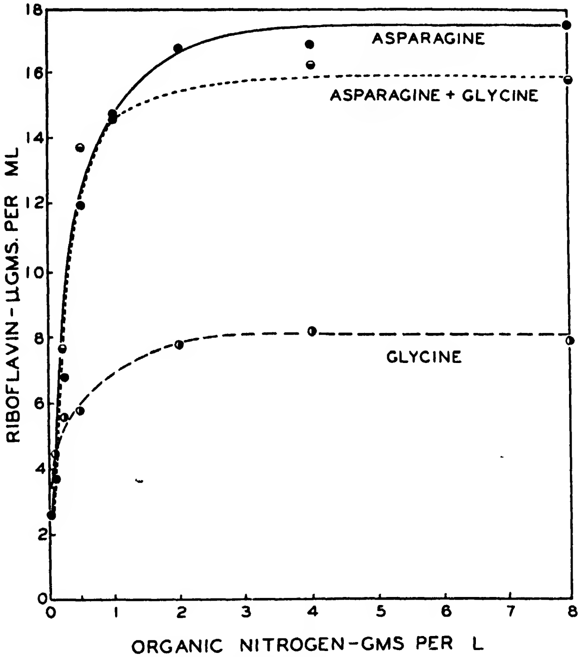

FIG. 5

Production of Riboflavin in Media Containing Varied Increments of Glycine, Asparagine, and Both of These Compounds in Equal Proportions

yield of vitamin B_2 in a glycine medium was increased from 8 to about 60 μg./ml. Hence it seems probable that by employing a favorable combination of circumstances, good yields might be obtained at a reasonable cost.

SUMMARY

Growth and production of riboflavin by a yeast have been studied in relation to varied conditions in synthetic culture media. The pro-

gressive accumulation of vitamin B_2 with time forms a sigmoid curve simulating a growth curve. The crop of yeast produced during a period of one week is not affected by the amount of the inoculum, but the final yield of riboflavin is greatly diminished by using inocula greater than about 12 mg. of moist yeast/100 ml. of medium.

Mechanical agitation was very effective in promoting growth and synthesis of riboflavin by the yeast.

The results obtained by varying the constituents in the medium indicate that distinct processes are involved in utilization of nutrients for growth and production of riboflavin. The yeast grew well in media containing arabinose, galactose, inulin, maltose, mannitol, sorbose, or xylose, but the yields of vitamin B_2 were relatively poor in cultures supplied with any one of these carbohydrates. Both growth and synthesis of riboflavin were satisfactory in media containing either dextrose, mannose, levulose, or sucrose as a source of carbon. Increments of galactose supplied in media containing dextrose increased somewhat the growth of the yeast, but greatly decreased production of the vitamin. Among the various nitrogenous compounds tested, asparagine and glycine are relatively inexpensive sources of organic nitrogen suitable for the production of riboflavin by this yeast.

Reference

(1) Burkholder, Paul R., *Proc. Natl. Acad. Sci. U. S.* **29**, 166–172 (1943).

BOOK REVIEWS

Vitamins and Hormones. Advances in Research and Applications. **Vol. I.** Edited by ROBERT S. HARRIS, Associate Professor of Nutritional Biochemistry, Massachusetts Institute of Technology, AND KENNETH V. THIMANN, Associate Professor of Plant Physiology, Harvard University. Academic Press Inc., New York, N. Y., 1943. XVII + 452 pp. Price $6.50.

The usefulness of a volume of this character consists in the authoritative and critical estimate which it provides of what is significant and important in each of the fields covered by the several chapters. Its merit depends largely upon the experience of the individual authors. The editors are to be congratulated on their success in enlisting eminent authorities as contributors as will be seen from the following abstract of the table of contents.

Choline—Chemistry and Significance as a Dietary Factor. BY C. H. BEST AND C. C. LUCAS.

The Appraisal of Nutritional Status. BY NORMAN JOLLIFFE AND RITA M. MOST.

Physical Methods for the Identification and Assay of Vitamins and Hormones. BY JOHN R. LOOFBOUROW.

The Chemical and Physiological Relationship Between Vitamins and Amino Acids. BY H. H. MITCHELL.

The Photoreceptor Function of the Carotenoids and Vitamins A. BY GEORGE WALD.

The Significance of the Vitamin Content of Tissues. BY ROGER J. WILLIAMS.

Growth-Factors for Protozoa. BY RICHARD P. HALL.

Physiology of Anti-Pernicious Anemia Material. BY GEORGE R. MINOT AND MAURICE B. STRAUSS.

The Intermediate Metabolism of the Sex Hormones. BY GREGORY PINCUS AND WILLIAM H. PEARLMAN.

The Hormones of the Adrenal Cortex. BY T. REICHSTEIN AND C. W. SHOPPEE.

A problem which editors face in gathering such a series of monographs into a single volume is that of securing comparable treatment and proper relative emphasis on each of the several topics. Other things being equal, the more eminent the authority the greater the danger of his preoccupation with other tasks, unless the editor is prepared to wait for a timely occasion. Opportunism is well advised in such a case for a forced schedule is almost certain to produce some hasty chapters among others more elaborate, a misfortune which seems to have been happily avoided in this instance. The several chapters are worthy of each other.

What such a volume of necessity lacks is unity of approach and singleness of

judgment. The latest advances in research can not yet have undergone a critical process of assimilation and selection by any one mind as is the case with older material, such as is presented in the masterly type of textbook or treatise. This difficulty has not been overcome in the present volume. The reader who could master the entire volume would have to be physiologist, chemist, physicist and physician. Few if any readers can judge equally well of the merits of the several contributions. The chapters are not written from a single point of view. A thread of chemistry which runs through them is their chief linkage. The book's greatest usefulness is, accordingly, as a work of reference rather than as steady reading.

Illustrative of the scope and thoroughness of the work is the bibliography of 326 references to choline at the end of the first section. Similar bibliographies are appended to the other sections.

The reviewer was gratified to encounter Dr. Wald's review of the chemistry of photoreceptor function which should prove a great convenience. Similarly the collected analysis of information on pernicious anemia factors appealed to him as timely. Dr. Loofbourow's collection of physical methods brings together for the analyst information otherwise requiring a wide-ranging search. The reviewer was relieved on examining Dr. Mitchell's summary on vitamins and amino acids to find that their "physiological relationships . . . are largely associative or indirect in character." It was comforting to know that Dr. Mitchell's more thorough examination of the matter did not reveal some unguessed significance.

A feeling of awe was engendered by 148 complex structural formulae of critical hormones and their derivatives as set forth by Reichstein and Shoppee. The wide range of the total subject matter of the book, coupled with the knowledge that several additional volumes will be required to summarize recent knowledge of all the vitamins and hormones, induced a perilous enlargement of the reviewer's bump of humility.

R. R. Williams, Summit, N. J.

Virus Diseases. By Members of the Rockefeller Institute for Medical Research: Thomas M. Rivers, Wendell M. Stanley, Louis O. Kunkel, Richard E. Shope, Frank L. Horsfall, Jr., Peyton Rous, Cornell University Press, Ithaca, N. Y., 1943. IX + 170 pp. Price $2.00 postpaid.

The book originated in six lectures which the authors delivered at Cornell University in the spring term of 1942. The lectures were endowed by Hiram J. Messenger, in 1923, who specified that the lectures should be "on the evolution of civilization, for the special purpose of raising the moral standard of our political, business, and social life."

In a brief foreword Thomas M. Rivers likens the realm of viruses and virus diseases to a boom town in which nothing is settled, and defines as the aim of the book the presentation of those facts about viruses which have been established and are of sufficient interest and importance.

The first lecture, by Rivers, deals with vaccinia, the best studied animal virus. Acquired immunity is first discussed and the author reviews the arguments for and reaffirms his belief in the persistence of virus in the host in those cases in which a lasting immunity results from a single infection. Next, the very complex problem

of the antigenic structure of vaccine virus is taken up and treated in historical order, leading up to the clarification of the relation between Craigie's soluble L and S antigens. The length of this discussion seems to the reviewer out of proportion with the importance of these findings. Finally physical structure and chemical nature of vaccine virus are discussed. The first of these has been investigated successfully by electron micrographs, which revealed a very peculiar structure and confirmed earlier estimates of its size. Chemical studies have yielded the most important results of all vaccine research, since they disclosed not only thymonucleic acid and protein but also *biotin*, *copper*, and *flavin-adenine-dinucleotide* as essential constituents of the virus. These findings justify indeed the hope, expressed by the author, that metabolic activities of the virus in the absence of living cells may yet be found.

The second lecture, by Stanley, deals with the best-investigated plant virus, tobacco mosaic virus, and with related strains, and centers around the problems of virus mutation. In the first part the author reports chemical analyses of eight related strains of tobacco mosaic virus. Significant differences between these strains in the content of certain amino acids and of sulfur were found. Comparing this result with the similarity of structure, revealed by X-ray analysis, the author suggests that the mutations "result from a diversion of the synthetic process by means of which a virus multiplies" and not from alterations of fully formed virus structures. The author postulates that "only those changes will occur which conform to the basic pattern of the given virus." This is an important hypothesis of the widest significance. The second part of this lecture is concerned with the interesting, but as yet unsuccessful attempts to produce mutations of tobacco mosaic virus by treating the virus chemically, so as to produce chemical derivatives of it. In all cases the virus grown after injection of chemical derivatives of it was indistinguishable from the original strain.

The third lecture, by Kunkel, deals with the experimental advantages to be derived from a study of a plant virus on different hosts. It describes the methods which have been developed to obtain transfer of virus from one host to another. The author's contention that new hosts in many cases were the key to progress in virus research is convincingly demonstrated. The peculiar difficulties encountered in many cases of virus transfer are intriguing, as, for instance, when it is desired to transfer to peach the virus of cranberries which causes false blossom. There exists an insect vector which normally carries the virus from cranberry to cranberry, but this animal, the blunt-nosed cranberry leafhopper, simply cannot stomach the peach. But these are mere technical difficulties. The real, and quite unsolved, problem seems to lie in the basic fact that many viruses are not transmissible by juice. This problem is stated, but no hypothesis is offered regarding the meaning of it.

The fourth and fifth lectures deal with exceedingly involved epidemiological problems. The swine influenza problem appears in large measure to be solved, and the presentation of the amazing solution in Shope's lecture is a model of thoughtfulness and clarity, and a brilliant story. The only obscure point in the solution appears to be the seasonal "masking" of the causative agent. The true meaning of this masking is not understood and its understanding may depend on the solution of problems of virus transmissibility in general.

The lecture on human influenza, by Horsfall, is little more than a compilation of recent work, most of which appears to be of inconclusive nature. It would seem that neither the subject matter of this lecture nor its method of presentation were suited to illustrate general problems of virus research.

The last lecture, by Rous, deals with the relation between viruses and tumors, and is a masterly review and analysis of the experimental results which have been obtained in this important field of research. To the reviewer there seem to exist obvious similarities in the problems treated by Kunkel and by Rous, namely with regard to the difficulties of proving the presence of a virus as the causative agent of an abnormal growth, in those cases where the abnormality cannot be transmitted by extracts of the abnormal tissue, but where the presence of a virus is nevertheless suspected. Neither of these two authors appears to take notice of the other's problems and the reader is left wondering what, if any, may be the reasons for this lack of reference to them. It would seem as if the lecturers, whose intent it was to present the reader with a comprehensive view of the entire field of virus research, did not attain this goal for themselves. Could it be that they were so engrossed in their own problems, they never heard or read each other's contributions?

From what has been said it may be seen that the book covers in small space pretty well the entire field of virus research in its manifold aspects, with the exception of bacterial viruses, and with the exception of virus interference, which is nowhere mentioned, although it has been shown to occur with animal, plant and bacterial viruses, and should be regarded as an important general fact about viruses. Otherwise, the important facts are everywhere brought out by the discussion of the best studied objects and they are presented by authorities in the respective fields who have contributed decisively to these fields. In most cases the presentation is lucid and concise. Though never dogmatizing, the authors permit the reader to become acquainted with those general ideas about viruses which are the "actuating causes" of the experiments. The Rockefeller Institute for Medical Research has given us in this little book a powerful demonstration of its dominant rôle in present day virus research. The reviewer is inclined to see an incongruity in this fact. Viruses are things of fundamental biological importance and of rights ought to be foremost in the hands and minds of biologists. Where would genetics be today if it were viewed as a field of medical research and were presented to us under the title: "Malformations and their inheritance?"

Every lecture is supplemented by an adequate bibliography; the attractive appearance and low price should add to the appeal of the book. It deserves the widest distribution among biologists.

M. Delbrück, Nashville, Tenn.

Advances in Enzymology, Volume II, edited by F. F. Nord and C. H. Werkman. Interscience Publishers, Inc., New York, 1942. 374 pp. Price $5.50.

The second volume of "Advances in Enzymology" continues the series which is published "to be of service to those investigators devoting their efforts to extending our knowledge in this field and related subjects; and to all who are interested in the realm of enzyme behavior." The editors have fully succeeded in their

aim by again selecting highly interesting topics, with outstanding specialists as authors. The fact that these men have devoted their time to contribute to this work is proof that the demand for a series like "Advances in Enzymology" is widely recognized. All the authors have been actively and successfully engaged in their respective fields, which makes some of the contributions rather ardent and personal. The speculative discussion, however, does not, as a rule, exceed permissible bounds.

Some of the chapters are written mainly for those investigators active in the field covered, especially the contributions of Max Delbrück on "Bacterial Viruses (Bacteriophages)," of Donald D. van Slyke on "The Kinetics of Hydrolytic Enzymes and their Bearing on Methods for Measuring Enzyme Activity," and of Max Bergmann on "A Classification of Proteolytic Enzymes." On the other hand, the article on "The Enzymatic Properties of Peptidases" by Marvin J. Johnson and Julius Berger will be found very informative by the reader who is less familiar with this subject.

The contributions on "The Chemistry of Tea-Fermentation" by E. A. Houghton Roberts, and on "Cellulose Decomposition by Microorganisms" by A. G. Norman and W. H. Fuller, are interesting in that they familiarize the enzyme chemist with these newer and less well-known fields. They may also help to stimulate further research along these lines.

Edgar J. Witzemann tries to reconcile much contradictory experimental data with theories in his presentation of "A Unified Hypothesis of the Reciprocal Integration of Carbohydrate and Fat Catabolism." Although the picture as visualized by him is a tentative or working hypothesis, it will be found very instructive.

C. H. Werkman and H. G. Wood have contributed an article on "Heterotrophic Assimilation of Carbon Dioxide." Needless to say, these two pioneers are masters of their subject. Those less familiar with the biochemistry of microorganisms will find the introductory part very helpful for a further understanding of the subject. A comprehensive review on the mechanism of carbon dioxide fixation by bacteria and by animal tissues has been urgently needed, and Werkman and Wood's contribution certainly fills this gap.

E. Albert Zeller gives an extensive review of the work dealing with "Diamine-Oxidase." Since this field is still in the very early stages of development, much of the data on specificity, affinity toward substrates, and inhibitors and activators will have to be revised as work on this enzyme progresses. Further research should shed more light on its biological significance, which is a subject of extensive discussion at present. As far as the therapeutic use of diamine oxidase is concerned, the author's reserve and skepticism concerning the practical application of the present crude preparations are gratifying.

The contribution of Hiroshi Tamiya on "Respiration and Fermentation of Aspergillus and Enzymes Involved in these Processes" offers delightful reading. The great practical importance of Aspergilli, their nutritional requirements, metabolism, and the products resulting from the latter are discussed. The thermodynamics of growth and metabolism are treated extensively, and the section on dehydrogenases bears witness to the impressive multiplicity of enzymatic reactions provided by nature in these relatively simple organisms. Numerous

unpublished observations of the author are reported and the extensive review of the literature which, in part, is scarcely available in most countries, makes this contribution a repertory of valuable information.

The chapters on "Vitamin K, Its Chemistry and Physiology" by Henrik Dam, and on "The Adrenal Cortical Hormones" by J. J. Pfiffner, might seem to be somewhat out of place in a book dealing with the advances of enzymology. It should be pointed out, however, that enzyme chemists will profit greatly from the information offered. Future work may even make possible an in vitro correlation of vitamin K as well as of the adrenal cortical hormones with enzyme systems. Pfiffner's treatise offers very edifying reading. The chapter on vitamin K, written by its discoverer, is probably the best review of this subject published so far.

F. SCHLENK, Galveston, Texas.

The Chemistry of Natural Coloring Matters. The Constitutions, Properties, and Biological Relations of the Important Natural Pigments. BY FRITZ MAYER, PH.D., formerly Professor of Chemistry in the University of Frankfort-on-Main; translated and revised by A. H. COOK, Ph.D., Department of Chemistry, Imperial College of Science, London, England. American Chemical Society Monograph No. 89. Reinhold Publishing Corporation, New York, N. Y., 1943, 354 pp. Price $10 00.

This book is based upon the second volume of the third German edition of Dr. Mayer's "Chemie der organischen Farbstoffe", published in 1935. In preparing the English text, Dr. Mayer made considerable revisions, adding new material to 1939. After the author's tragic death further additions were made by his colleague and translator, Dr. Cook, covering the literature to August 1941.

Among the naturally occurring pigments which have been structurally characterized, so many types of organic compounds occur that logical classification is a matter of some difficulty. This book is divided into five chapters: Carotenoids; Diaroylmethane Compounds; Carbocyclic Compounds; Heterocyclic Compounds; and Compounds Containing Heterocyclic Nitrogen Atoms. As chapter four deals exclusively with compounds containing heterocyclic oxygen atoms, this might, with advantage, have been indicated in the chapter heading. The literature of natural product chemistry is cluttered with descriptions of imperfectly characterized compounds, which may, or may not be new. Drs. Mayer and Cook have included only coloring matters which have been characterized by analytical figures and significant chemical properties. The section on each compound is headed by the name of the compound (in bold face type) followed by a description of its occurrence, properties, evidence of structure, and relationship to other pigments of the same chemical group. The layout of the text makes it very easy to find any particular pigment, and extensive references to original publications together with an excellent subject index make this a most valuable reference book.

The reviewer is more familiar with the carotenoid pigments than the other groups discussed in subsequent chapters. A careful study of this section fails to reveal any serious grounds for criticism. On page 15 the coloring matter of the saffron is described as a digentiobioside, *crocetin*, where *crocin* is obviously intended, and, on the same page, it is stated that astaxanthin is a carotenoid bound

to a protein. Actually astaxanthin is the prosthetic carotenoid which can be detached from a chromoprotein-complex. Both crocin and astaxanthin are correctly described elsewhere in the text. The omission of any reference to vitamin A_2 under the section dealing with vitamin A calls for comment, as also does the omission of the work of Wald and others on the part played by carotenoid pigments in the retinal cycle.

Chapter two occupies three pages only, describing a single diaroylmethane pigment, curcumin. The third page of this chapter is erroneously headed "Carotenoids" instead of "Diaroylmethane Compounds."

Chapter three is devoted mainly to a discussion of quinonoid pigments of various types; chapter four includes the flavone, isoflavone and anthocyanine families of pigments, and detailed surveys of pyrrole and alloxazine pigments occupy most of chapter five.

The few criticisms made above are of a very minor character, and this book fully maintains the high standards expected of American Chemical Society Monographs.

R. NORMAN JONES, Kingston, Canada.

Textbook of Biochemistry. By BENJAMIN HARROW, Ph.D., Professor of Chemistry, City College, College of the City of New York. Third edition. ix + 537 pp. 118 illustrations. 15 x 23 cm. Philadelphia and London: W. B. Saunders Co. 1943. $4.00.

The third edition of Harrow's *Textbook of Biochemistry* follows closely the plan of the previous editions. Somewhat enlarged (537 pages as compared with 439 for the second edition), it contains numerous small additions on new developments, as well as a new chapter on immunochemistry and chemotherapy. The chapters on vitamins and hormones, the longest in the book, have been particularly expanded. New work on the use of isotopes in the study of metabolism has been systematically covered. Included also are the enriching of foods and nutritional problems of the war period; electron micrographs in the study of viruses; blood plasma and the war; induction of cancer by chemical compounds, and other recent topics of general interest.

As a whole the book is a very effective presentation of the subject. It is concise, yet very comprehensive; the style is lucid and the illustrations and examples are well chosen and convincing. Recent advances are well represented. The selected references at the end of each chapter provide a good evaluation of the literature.

Physico-chemical topics are, as a rule, very inadequately treated. The molecular weights of proteins, the Donnan membrane theory, and the concept of oxidation-reduction potentials are mentioned; however, without exposition of the experimental methods and observations by which the ideas were developed, or of their significance. At times recent developments are included as an obvious afterthought, when they might better have been given in the original treatment of the subject. Thus under the heading of forms in which CO_2 exists in the blood, carbonic acid and $NaHCO_3$ only are considered significant, while five pages later a paragraph is devoted to carbamino compounds. In the original discussion of pH determination, it is stated that two methods are available, indicator and potentio-

metric; and one gains the impression that these only are significant. Later the use of the ionization constant equation for pH determination is described in detail.

On the whole, the book is remarkably free from errors and misstatements. A few, however, since they are repeated from previous editions, should perhaps be noted here. To write equilibrium arrows between α- and β-methyl glucoside, in the same way as they are written between α- and β-glucose, is very misleading. The statement that the reaction of α- glucose with boric acid suggests that the two OH groups on carbons 1 and 2 are "on the right" is a naive oversimplification; it does indicate that they have a similar orientation. The statement that the individual amino acids *all* show powerful specific dynamic action effects is not borne out by experiment. In discussing the *increased* alkali reserve following alkali feeding, it is erroneously stated that the same condition may arise from overventilation; as a matter of fact, overventilation, while elevating the blood pH, results in a *decreased* alkali reserve. The author continues to misspell the name *Hasselbalch*.

In a 1943 book where recent developments are as a rule so well included, it is surprising to find some 1942 developments which are omitted. Under the chemistry of biotin, the structural formula is not given, and the discussion of carbonic anhydrase does not include the isolation of this enzyme in crystalline form.

Because it is concise, up-to-date, comprehensive, and readily understandable, the book is recommended as a valuable addition to our textbooks of biochemistry.

University of Chicago MARTIN E. HANKE, Chicago, Ill.

Elements of Food Biochemistry. By WILLIAM H. PETERSON, Professor of Biochemistry in the University of Wisconsin, JOHN T. SKINNER, Assistant Chemist of Kentucky Agricultural Experiment Station, and FRANK M. STRONG, Associate Professor of Biochemistry in the University of Wisconsin. Prentice-Hall, Inc., New York, N. Y. 1943. xii + 291 pp. Price $3.00.

This is primarily a text book for those interested in the biochemical aspect of food and nutrition. It should prove valuable to those who have had some initiation in biochemistry and organic chemistry. The book is decidedly not a text in "kitchen chemistry."

It is broken down into nine chapters, an appendix of Tables showing the proximate composition, mineral and vitamin content of numerous foodstuffs, and a subject index. Considerable portion of the data on vitamin content of foods has not previously been published. No references are given to individual statements, although selected bibliography of well known texts and monographs is provided at the end of each chapter. Well chosen and carefully considered questions close each chapter to provide the student some orientation and points of stress.

The material is organized along well accepted logical lines, and is presented in clearly formulated short paragraphs. The economic aspect of food and nutrition as well as the industrial development of the manufacture of foodstuffs and accessory food factors is very interestingly interwoven with the entire subject. In view of the importance of economics and its decisive influence upon the practical aspects of the science of nutrition, this appears to be an unusual and a welcome note in the presentation of the subject for public education. It is only to be desired that present day political scientists and economists with any influence upon the future

development of human relationships will begin seriously studying the elements of nutrition.

The material covered by the book is brought up to date by inclusion of more or less recently discovered biological phenomena and methods of research, such as the mechanism of transamination, the use of radioactive elements as metabolic "tracers," etc. The consideration of a rather well substantiated mechanism of transmethylation, on the other hand, is omitted entirely, and the treatment of the interrelationship between choline, methionine, creatinine and the lipotropic action of the "labile methyl group" is brief to the point of obscurity. This omission explains, perhaps, the presence of a statement that 18 per cent of casein in the diet of a growing rat is considered adequate. The well grounded work of du Vigneaud, Griffith, and others on the importance of adequate amounts of "labile methyl group" in the diet of the rat suggests the need for a considerably higher level of casein if the latter is fed as the sole source of protein and the "labile methyl group" in an otherwise adequate diet.

"Elements of Food Biochemistry" is a welcome addition to a rather short list of good texts on nutrition which are suitable for class use.

JAKOB A. STEKOL, New Orleans, La.

Reports of the Biochemical Research Foundation of the Franklin Institute. Vol. VI, 1940–1941. Distributed gratis to institutional libraries. The Biochemical Research Foundation, Newark, Delaware.

This volume of about 350 pages represents a collection of reprints of publications from the laboratories of the Foundation, Director, Dr. Ellice McDonald, for the biennium 1940–1941. The reprints fall into two groups: one of 24 regular scientific papers (numbered 166–189), published in 12 different journals by 21 different authors, most of them appearing in the Journal of Biological Chemistry, Journal of Bacteriology, and journals of the American Chemical Society. The second group represents NOTES published originally in a special section of the Journal of the Franklin Institute. The NOTES include abstracts of papers from the laboratories, reports of Foundation seminars given by invited guests and in addition 20 original items usually of limited length. The scope of the topics is rather wide, but a number of noteworthy articles deal with various bacteriological subjects, particularly with reference to soil bacteria. Several papers are concerned with enzyme problems, and another group with various aspects of the physical chemistry of big molecules. The latter is especially prominent among the original items in the NOTES. The volume also includes the annual reports of the director. During the period covered by the volume, the institution moved from Philadelphia to a new laboratory in Newark, Delaware, but the disturbance incidental to moving did not show itself in the productivity of the laboratory.

E. O. KRAEMER, NEWARK, DEL.

Conservation of Scholarly Journals

The American Library Association created in 1941 the Committee on Aid to Libraries in War Areas, headed by JOHN R. RUSSELL, the Librarian of the University of Rochester. The Committee is faced with numerous serious problems and hopes that American scholars and scientists will be of considerable aid in the solution of one of these problems.

One of the most difficult tasks in library reconstruction after the first World War was that of completing foreign institutional sets of American scholarly, scientific, and technical periodicals. The attempt to avoid a duplication of that situation is now the concern of the Committee.

Many sets of journals will be broken by the financial inability of the institutions to renew subscriptions. As far as possible they will be completed from a stock of periodicals being purchased by the Committee. Many more will have been broken through mail difficulties and loss of shipments, while still other sets will have disappeared in the destruction of libraries. The size of the eventual demand is impossible to estimate, but requests received by the Committee already give evidence that it will be enormous.

With an imminent paper shortage attempts are being made to collect old periodicals for pulp. Fearing this possible reduction in the already limited supply of scholarly and scientific journals, the Committee hopes to enlist the cooperation of subscribers to this journal in preventing the sacrifice of this type of material to the pulp demand. It is scarcely necessary to mention the appreciation of foreign institutions and scholars for this activity.

Questions concerning the project or concerning the Committee's interest in particular periodicals should be directed to DOROTHY J. COMINS, Executive Assistant to the Committee on Aid to Libraries in War Areas, Library of Congress Annex, Study 251, Washington, 25, D. C.

Elmer Otto Kraemer

1898–1943

Elmer Otto Kraemer, head of the Physical Chemistry Division of the Biochemical Research Foundation of the Franklin Institute at Newark, Delaware, and an Editor of "Archives of Biochemistry," died September 7, 1943, at the American Chemical Society meeting in Pittsburgh. He was an internationally recognized authority on the colloid chemistry of large molecules.

Dr. Kraemer was born at Liberty, Wisconsin, on February 27, 1898. He received his B.S. degree at the University of Wisconsin in 1918. He spent 1921–1922 as a Fellow of the American Scandinavian Foundation with Svedberg at Upsala, Sweden, and the following year with Freundlich at the Kaiser Wilhelm Institut. In 1923 he returned to the University of Wisconsin and received the Ph.D. degree in 1924.

After a year as National Research Fellow, he was appointed Assistant Professor of Colloid Chemistry at Wisconsin. When the du Pont Company started its program of fundamental research in organic and physical chemistry in 1927, Dr. Kraemer became the Colloid Group Leader in the Chemical Department. He served with distinction in this position until 1938, when he was awarded a Lalor Foundation Fellowship to continue his ultracentrifugation studies on giant molecules in Svedberg's laboratory. When he was forced to return to this country at the end of 1939 because of the international situation, he accepted the directorship of physical chemical research on bio-colloids at the Biochemical Research Foundation. Since 1940 he has also been special lecturer in colloid chemistry at the University of Delaware.

Dr. Kraemer was the author of numerous technical papers, of the well known chapter on colloids in Taylor's "Treatise on Physical Chemistry," of two chapters in "The Ultracentrifuge," by Svedberg and Pedersen, and of two chapters in "Chemistry of Large Molecules," a series of lectures given at Western Reserve University. He helped to organize the Society of Rheology and served as Associate Editor of the Journal of Rheology during the life of that publication. From 1933 to 1935 he was Associate Editor of the Journal of Chemical Physics. He was on the

editorial board of the Interscience series of monographs on "High Polymeric Substances" and was the principal editor of "Recent Advances in Colloid Science." Dr. Kraemer held membership in the American Chemical Society, the American Association for the Advancement of Science, the Society of Rheology, the Franklin Institute, the New York Academy of Sciences, and formerly in the Kolloid Gesellschaft.

The editors and publishers of the "Archives of Biochemistry" remember with gratitude Dr. Kraemer's faithful service during the term of his editorship. They count his passing an irreparable loss.

Studies on Plant Tumors. Part II.

Carbohydrate Metabolism of Normal and Tumor Tissues of Beet Root[1]

Arthur Charles Neish[2] and Harold Hibbert

From the Division of Industrial and Cellulose Chemistry, McGill University, Montreal, Canada

Received August 19, 1943

Introduction

Various workers including the authors have made comparative chemical analyses of the tumor and normal tissue of plants inoculated with *Phytomonas tumefaciens*, the results obtained by them being in general agreement (1). From the data it is evident that the tumors rob the contiguous normal tissue of stored sucrose and use it for the synthesis of protoplasm and cell wall material (1). Growth in these tumors is a result of increase in cell number rather than increase in cell size and in this respect they resemble the malignant neoplasms of animals.

No previous studies have been reported on metabolic changes in plant tumor slices and indeed very little work of this kind has been done on any tissue of the higher plants.

This paper is a report on a comparative study of the metabolism of tumors produced by *P. tumefaciens*, and the normal tissue of the *same* beet root. The work was undertaken in order to obtain data on the metabolism of higher plants and particularly to compare the metabolism of a rapidly-growing plant tissue with that of a mature storage tissue no longer capable of rapid growth.

Experimental

Analyses were made to ascertain the amount of sugar metabolized by surviving tissue slices, (a) under aerobic conditions (respiration);

[1] This work forms part of a thesis presented by A. C. Neish to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Holder of a Fellowship from the National Research Council of Canada.

(b) anaerobic conditions (fermentation); and (c) during an aerobic following an anaerobic period in order to account for the sugar so metabolized. Essentially, then, this work is a comparative analytical study of respiration, fermentation, and the Pasteur Effect in surviving slices of the normal and tumor tissues of the same beet root as they metabolize their stored sucrose.

Experimental Tissue Material

The tumors (crown galls) induced by *Phytomonas tumefaciens* are easily produced experimentally and form very satisfactory material for studies on the metabolism of a rapidly-growing plant tissue. The possible objection that use of this material implies measurement of the metabolism of the bacteria as well as that of the host tissue is not valid since the bacteria form a very small proportion of the total mass of the tissue and are found chiefly on the surface of the tumor and are removable by a stream of running water (2). Since the tissues were thoroughly washed and the experiments of short duration it is very unlikely that any bacteria present could produce a measurable effect. Actually a linear relation was found between time and oxygen absorption by slices of tumor tissue indicating improbability of aerobic bacterial development to any measurable extent during the course of the experiments.

Beets of the variety "Extra Early Flat Egyptian" were grown in pots in a green house. Cultures of the bacterium *Phytomonas tumefaciens* were made on beef peptone agar or potato-dextrose nutrient agar. When the beet roots had reached a diameter of 1–2 cm. they were inoculated on one side of the crown by lacerating with a needle coated with bacteria from a two day old culture. The tumors developed at the point of inoculation, and sometimes exceeded in size the remainder of the beet root. Time to maximum growth for use was four to six weeks. If kept longer the tumors developed secondary infections rendering them unfit for use.

The fleshy part of the root, including the tumor, was scrubbed in a stream of running water with a test tube brush to remove all adhering soil. The tumor was then separated from the normal tissue by cutting along a line which followed the normal contour of the root while a sample of normal tissue was taken from the area diametrically opposite the tumor. All tissues were sliced free hand using a Durham Duplex razor blade, the material so prepared giving quite consistent results with regard to thickness of slice.

Analytical Studies on Metabolism

In order to harmonize differences due to variation between individual beets, composite samples were used. The tissues were cut into blocks about 0.8 to 1 cm. square and the latter into slices 1 mm. (about 8 cells) thick. These were washed with sterile water in a beaker, and thoroughly mixed in the process. The water was decanted off and the excess surface water removed by pressing the slices between filter papers. From this composite material samples (15 g.) were weighed out, and since each contained about 100 individual slices they could be considered to have essentially the same composition.

One sample was frozen immediately at $-70°C.$, ground in a porcelain mortar, with quartz sand, under distilled water, and the aqueous extract reserved for analysis.

The other samples were allowed to metabolize for a period of several hours under aerobic or anaerobic conditions and then extracted in the same manner as previously. Comparison of the analytical data would thus show the metabolic changes which had occurred during this period.

The metabolism took place under a stream of sterile CO_2 free air, or pure nitrogen, in an apparatus analogous to that used for the determination of uronic acids (3). This permitted the measurement of the carbon dioxide evolved, using relatively large tissue samples. The metabolism vessel was a 250 ml. round bottom flask containing 30 ml. of phosphate buffer, at pH 6–8, and fitted with a rubber stopper containing a fine tipped glass inlet tube reaching below the surface of the liquid. A stream of air or nitrogen (free from carbon dioxide and bacteria) was drawn through the system and the carbon dioxide evolved measured by absorption in standard barium hydroxide as in the determination of uronic acids (3). In all experiments the medium containing the tissue slices was maintained at a temperature of 28°C.

Analytical Methods

Sucrose, glucose, and fructose were estimated (4) using diastase, takadiastase, and HCl for inversion of the cleared extract. Since all three catalysts gave the same result with these extracts HCl was used in most cases. Inorganic phosphate (5), starch (6), oxalic, malic and citric acids (7), keto acids (8), acetaldehyde (9), lactic acid (9, 10) and ethanol (11) were also determined. Ester phosphorus was measured by the increase in organic phosphorus on hydrolysis by *N* HCl at

100°C. for one hour and total reactive carbonyl by a direct bisulfite titration of the total aqueous extract (9). Ascorbic acid was determined by reduction of a trichloroacetic extract with H_2S, removal of the latter by CO_2 and titration with a 2,6-dichlorophenol-indophenol solution standardized against pure ascorbic acid.

Manometric Measurements

The oxygen consumption and respiratory quotient were measured using the apparatus and technique of Dixon and Keilin (12). The medium employed was a phosphate buffer at pH 6.8; the thermostat was kept at 28°C. About 6 slices of tissue, 1.0 mm. thick, and corresponding to 0.5 to 1.0 g. of fresh tissue, were used.

Results

1. *Respiration of Beet Tissue Slices*

Rate of Respiration

Manometric measurements of the rate of oxygen consumption showed that the pH of the medium had no effect within the range pH 5.5 to 8.0, but at more extreme values the rate was decreased due to tissue injury as evidenced by diffusion of the red pigment from the cells. A phosphate buffer (pH 6.8) was employed in all subsequent experiments. Tissue slices (1.0 mm. thick) were found to remain alive in this medium for 12 hrs. (tumors) and 20 hrs. (normal tissue) after which they died, as shown by plasmolytic tests. The length of life of tumor slices could be doubled by addition of sucrose (1%) to the medium, but this encouraged the development of microorganisms. Duration of the individual experiments was less than 10 hrs., and addition of sucrose was omitted.

Slicing the beet root tissue caused a marked increase in rate of oxygen consumption, an effect also observed previously in the case of carrots (13). Rate of respiration of tumor tissue increased with decrease in slice thickness up to a minimum thickness of 1.0 mm. (Fig. 1). Below this the rate of respiration decreased, presumably due to the proportionately larger number of cells injured by slicing.

Tissue slices 1.0 mm. in thickness were used in all subsequent experiments.

Tumor slices respire two to three times as rapidly as an equal weight of normal tissue slices (Table I), but if this comparison is made on the basis of equal amounts of protein (13) the two types of tissue have

about the same rate of oxygen consumption. The presence of a tumor seems to lower the rate of respiration of the normal tissue by about 20% (Table I).

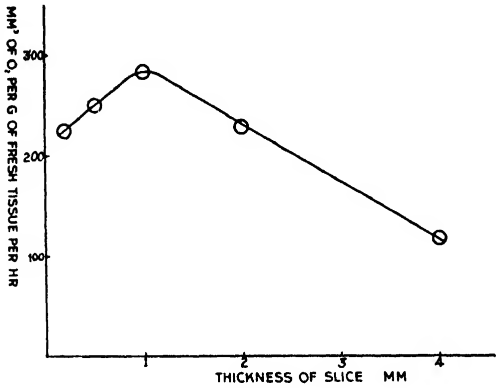

FIG. 1
The Effect of the Thickness of Slice of Tumor Tissue on the Rate of Respiration

TABLE I

Rates of Respiration of Beet Tissues

mm.^{3} of O_2 absorbed per g. of fresh tissue per hr.

	Tumor Tissue	Normal Tissue of Tumorous Beet	Tissue of Healthy Beet
	270	70	105
	275	105	120
	280	98	115
	310	88	135
	270	80	80
	240	71	125
			75
Average value..........	274	86	108

Plasmolysis of slices of both types of tissues in sucrose solutions shows that there is a close relation between the rate of respiration and the water content (Table II). There is no effect until plasmolysis is noticeable; after that the rate of respiration is decreased as water is withdrawn from the cells. This is a reversible effect since the cells regain their original rate of respiration when deplasmolyzed.

Metabolism of the Sugar Respired

An interesting difference was found between the tumor and normal tissue with regard to the respiratory quotient (R.Q.) The R.Q. for tumor and normal tissue was 0.92 and 0.64 respectively, indicating

TABLE II

Effect of Plasmolysis on Rate of Respiration

Conc. of Sucrose in the Medium	mm.^{3} of O_2 absorbed per g. of fresh tissue per hr. Tumor tissue	Normal tissue of tumorous beet	Remarks
0.2 *M*	311	83	No plasmolysis
0.6 *M*	311	83	Slight plasmolysis in tumors
1.0 *M*	212	60	Noticeable plasmolysis
1.5 *M*	175	56	Marked plasmolysis
2.0 *M*	137	34	Strong plasmolysis
0.2 *M* (after 2.0 *M*)	275	85	Cells deplasmolysed

TABLE III

*Formation of Malic, Citric, and Oxalic Acids by Respiring Beet Tissue Slices**

mg. per 100 g. of fresh tissue

	Tumors		Normal Tissue of Tumorous Beet	
	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism	Initial amount	Amount after 3 hrs. of Aerobic Metabolism
Oxalic Acid	285	288	78	108
	266	262	68	85
Malic Acid	132	137	195	257
	130	149	195	244
Citric Acid	218	185	56	81
	263	230	90	116

* 5 g. composite samples of tissue slices (1.0 mm. in thickness) were shaken in 10 ml. of phosphate buffer at pH 6.8 and 28° C. for 3 hrs.

that the respiration of these tissue slices is not exclusively complete oxidation of carbohydrate to carbon dioxide and water. The low R.Q. of the normal tissue is explained by formation of citric, malic, and oxalic acids from sucrose (Table III). Quantitative calculations from

these data show that the amounts of these three acids account for about 75% of the oxygen consumed in excess of that required for an R.Q. of unity. Such acid formation does not occur in tumors to any marked extent indicating a marked difference in the metabolism of the two types of tissue.

Table IV contains the averaged results of a considerable number of experiments made in an attempt to account quantitatively for the total carbohydrate metabolized during a 3-hour period of respiration. The balance is far from complete for tumors, in that while 25% of the sugar

TABLE IV

Carbon Balance Sheet for the Aerobic Metabolism of Carbohydrate in Tumors, and the Normal Tissue of Tumorous Beets

mg. (as carbon) per 100 g. of fresh tissue

	Tumors		Normal Tissue of Tumorous Beet	
	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism
CO_2 evolved		34.1		12.0
Total reducing sugars after inversion	526	391	914	845
Acetaldehyde	1.1	2.3	0.4	0.8
Ketoacids (as pyruvic acid)	11.6	10.5	7.5	10.5
Ascorbic acid	8.1	4.1	5.7	3.3
Oxalic acid	74	74	19	26
Citric acid	82	77	27	37
Malic acid	44	48	65	84

metabolized is accounted for by the CO_2 evolved, the fate of the remainder is unknown. In normal tissue the CO_2 evolved accounts for 19% of the sugar metabolized while the combined oxalic, malic, and citric acids represent a further 52%; a total of 71%.

These results are reported on a *fresh weight* rather than on the more conventional *dry weight* basis. However, since both types contain about 10% solid matter, a value found to remain nearly constant in various tissue samples, either fresh or dry weight can be used as a basis for calculating analytical results. An average value for the dry matter content of tumor and normal tissue is 10.4% and 9.8% respectively.

Inhibitors of Respiration

The effect of such substances as sodium cyanide, fluoride, iodoacetate, and malonate, phenol, hydroxylamine, cyclohexanone, and sodium bisulfite on the rate of respiration was determined by use of the Dixon-Keilin apparatus (12). The rate was measured, the inhibitor added from the side arm and the rate again ascertained after allowing a period of thirty minutes for attainment of equilibrium, and the percentage inhibition then calculated.

TABLE V

Inhibition of Respiration of Beet Tissue Slices

	Tumors	Normal Tissue of Tumorous Beet
Per cent Inhibition by NaCN		
0.01 *M*	81	90–100
0.03 *M*	87	90–100
Per cent Inhibition by NaF		
0.003 *M*	nil	nil
0.066 *M*	24	76
0.333 *M*	70	90–100
mg. of Ester Phosphorus per 100 g. of Fresh Tissue*		
0.003 *M* NaF	nil	nil
0.333 *M* NaF	{12.5, 12.9}	{15, 15}
Per cent Inhibition by $NaHSO_3$		
0.0024 *M*	nil	nil
0.0096 *M*	78	68
0.0317 *M*	100	100

* Amount present after metabolizing in presence of the specified concentration of NaF for 12 hrs. at 25° C. in phosphate buffer at pH 6.8.

Evidently (Table V) the normal tissue is more sensitive to cyanide and fluoride poisoning than the tumor. Due to the low rate of oxygen consumption by the normal tissue and the high density (4.0) of the manometer fluid, the method was not sensitive enough to measure, in the case of normal tissues, a respiration value less than 10% of the original rate. Nevertheless, distinct differences were noticeable between the two types of tissue, especially with fluoride, where phosphate esters also accumulated in the poisoned tissues when the concentration

of the inhibitor was great enough to cause a marked decrease in the rate of oxygen consumption (Table V). This indicates that phosphorylations may play an important rôle in the metabolism of these tissues, particularly with the normal tissue.

Although it requires a rather high concentration of these poisons to affect, seriously, the respiration of these tissues, the inhibition observed is not due to killing of the tissues since plasmolytic tests showed them to be alive at the end of the experiment.

Various other substances were tried in an attempt to differentiate between respiration of tumors and normal tissues though without much success. Malonate, even in 0.1 *M* concentration, exerted no effect, and the fact that aqueous extracts of frozen beet tumor and normal tissue were lacking in ability to oxidize succinic acid makes it unlikely that respiration in beet tissues involves the dicarboxylic acid system of Szent-Györgyi (15).

It was found, quite unexpectedly (Table V), that sodium bisulfite was capable of completely inhibiting the respiration of both tumor and normal tissue. Other carbonyl group reagents such as hydroxylamine (1%), cyclohexanone (0.3 *M*), *m*-nitrobenzoyl hydrazine (4%) and 2,4-dinitrophenyl hydrazine (saturated) were without effect on the rate of respiration hence it is unlikely that the effect of bisulfite is due to its affinity for the carbonyl groups of intermediary metabolites.

Metabolism of Bisulfite Poisoned Tissues

A comparison of the aerobic carbohydrate metabolism of tissue slices in the presence and absence of bisulfite shows no striking difference between tumors and normal tissue (Table VI). Metabolism still occurs even though the bisulfite is present in sufficient concentration to completely inhibit oxygen consumption. The bisulfite decreases the rate of metabolism (as measured by sugar utilized or carbon dioxide evolved) to 25% of the original value in tumors and 50% in normal tissue and also inhibits ascorbic acid metabolism and causes accumulation of a considerable amount of bisulfite-binding substances reported as total reactive carbonyl (Table VI). An attempt to identify the substances included in this category was not entirely successful. Acetaldehyde accounts for about 10% of the reactive carbonyl but this is offset by the decrease in keto acids that occurs in bisulfite poisoned tissues, so that it does not help to account for the increase in reactive carbonyl that occurs in the presence of bisulfite.

Tests for acetoin (16) and methyl glyoxal (17) yielded negative results. Spoehr and Strain (18) showed that glyceraldehyde is con-

TABLE VI

Aerobic Carbohydrate Metabolism of Bisulfite Poisoned Beet Tissues

mg. per 100 g. of fresh tissue

	Tumors			Normal Tissue of Tumorous Beet		
	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism in absence of bisulfite	Amount after 3 hrs. of Aerobic Metabolism in presence of 0.033% bisulfite	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism in absence of bisulfite	Amount after 3 hrs of Aerobic Metabolism in presence of 0.033% bisulfite
CO_2 evolved		152 154	41 35		57 51	31 28
Total reducing sugars	524 489	546 490	604 562	137 112	228 212	196 195
Total reducing sugars after inversion	1247 1385	921 1038	1168 1283	2357 2209	2137 2098	2255 2114
Acetaldehyde	2.9 1.4	4.2 4.4	14.0 13.2	1.4 nil	1.4 1.4	5.6 4.4
Keto acids (as pyruvic acid)	25 33	21 32	12 13	12 26	21 32	10 15
Ascorbic acid	21 19	7 13	21 19	13 12	7 9	17 12
Glyceraldehyde	5	5	54	6	6	33
Total reactive carbonyl	12 15	18 17	90 88	7 6	7 7	42 36

verted to methyl glyoxal by boiling for 15 mins. in an acid solution containing 1% aniline, and this reaction was used to estimate the glyceraldehyde present. The amount of methyl glyoxal evaluated

by a colorimetric method (19) showed that a tenfold increase in glyceraldehyde content occurred when the tissues were poisoned by bisulfite.

The data in Table VI were obtained by a large scale gravimetric adaptation of this same method which involves isolation of the methyl glyoxal as the 2,4-dinitrophenyl osazone.

This method was applied to trichloroacetic acid extracts of these tissues, freed from bisulfite by a stream of carbon dioxide. It was not practicable to separate methyl glyoxal from the solution by distillation since even pure sucrose solutions, on distillation with bicarbonate, yield distillable methyl glyoxal in 0.1% yield. It was identified by m.p. of the 2,4-dinitrophenyl osazone, recrystallized from nitrobenzene, and mixed m.p. determination (293°C., uncorrected). Since no phosphate esters accumulated in the presence of bisulfite the glyceraldehyde which was formed could not have been phosphoglyceraldehyde.

2. *Fermentation and the Pasteur Effect*

Differentiation of Fermentation and Respiration

Phenol (0.033 *M*) caused a 25% aerobic acceleration of oxygen consumption in both tissues while under anaerobic conditions the carbon dioxide output was decreased by 33% in normal and 67% in tumor tissue. Sodium monoiodoacetate (0.033 *M*) had no effect on respiration during the first hour, but after two hours the rate decreased 60–65% in both cases. Under anaerobic conditions the same concentration of iodoacetate immediately decreased the rate of CO_2 output by 90% in tumors and 70% in normal tissue.

This apparent differentiation between respiration and fermentation may be due to the greater permeability of the tissues under anaerobic conditions, as suggested by Turner's work (20) on the toxicity of iodoacetate towards carrot root slices. It is also possible to interpret results of this nature as proving the inaccuracy of the Unitary Theory of the Pasteur Effect (20, 21).

Anaerobic Metabolism of Carbohydrate and the Pasteur Effect

Analyses were made to determine the changes occurring in the tissue slices during a reaction period under nitrogen and also the effect of admitting air to such fermenting tissue slices. The averaged results for two or more experiments are shown in Table VII (tumor slices) and Table VIII (normal tissue slices) where an attempt is made to account quantitatively for the sugar metabolized under these conditions.

TABLE VII

Carbon Balance Sheet for the Anaerobic Carbohydrate Metabolism and Subsequent Aerobic Metabolism of Beet Tumor Slices

mg. (as carbon) per 100 g. of fresh tissue

	Initial Amount	Amount after 6 hrs. of Anaerobic Metabolism	Amount after 6 hrs. of Anaerobic Metabolism followed by 4 hrs. of Aerobic Metabolism
CO_2 evolved		42.8	99.9
Sucrose	552	378	143
Glucose	47	62	40
Fructose	52	54	50
Starch	26	25	24
Ethanol	18	92	99
Acetaldehyde	nil	0.6	0.8
Keto acids (as pyruvic acid)	5.1	7.1	8.5
Lactic acid	10	12	10
Malic acid	12	14	8
Citric acid	105	86	58
Oxalic acid	136	136	134
Reactive carbonyl	23	29	27

TABLE VIII

Carbon Balance Sheet for the Anaerobic Carbohydrate Metabolism and Subsequent Aerobic Metabolism of Slices of the Normal Tissue of Tumorous Beets

mg. (as carbon) per 100 g. of fresh tissue

	Initial Amount	Amount after 5 hrs. of Anaerobic Metabolism	Amount after 5 hrs. of Anaerobic Metabolism followed by 3 hrs. of Aerobic Metabolism
CO_2 evolved		19.2	46.5
Total reducing sugar after inversion	1683	1371	1470
Starch	12	10	10
Ethanol	12	51	61
Lactic acid	nil	76	30
Acetaldehyde	nil	nil	0.3
Malic acid	8	11	7
Citric acid	32	27	24
Oxalic acid	37	26	30
Keto acids (as pyruvic acid)	3.4	5.2	8.0
Reactive carbonyl	6	12	14

An alcoholic fermentation occurs in tumor tissue slices and accounts for 70% of the sugar metabolized. The observed ratio of carbon

dioxide carbon to ethanol carbon is 0.58 being fairly close to the theoretical figure of 0.50. There is no detectable lactic acid production although a small increase in keto acids occurs.

When air is admitted ethanol production is inhibited, but metabolism of sugar continues at an increased rate, only 23% of it being accounted for, as evolved CO_2. Citric acid is metabolized simultaneously with the carbohydrate under these conditions. During the "recovery" period 127 mg. of oxygen were consumed per 100 g. of fresh tissue. This corresponds to an R.Q. of 1.17 which, when corrected for the fermentation occurring during this period, becomes 1.10.

The metabolism of normal tissue under these conditions (Table VIII) is quite different from that of the tumor. Both lactic acid (24%) and alcoholic fermentations (19%) occur, and together account for 45% of the sugar metabolized under anaerobic conditions. The observed ratio of carbon dioxide carbon to ethanol carbon is 0.51, being very close to the theoretical value of 0.50 for an alcoholic fermentation. When air is admitted about one-third of the sugar fermented is resynthesized to sucrose while 40% of the lactic acid disappears and could be utilized in this process. It is also interesting to note that the 5 hr. period of anaerobiosis has modified the tissue so that it no longer converts carbohydrate to oxalic, malic, and citric acids. This is also reflected in the R.Q. for the recovery period which is 1.73 while that of the normal tissue before being subjected to a period of anaerobiosis is 0.64. The R.Q. for the recovery period, after correction for the alcoholic fermentation which has occurred during the period, is 1.17.

Normal and tumor tissues thus are differentiated by the existence of a lactic acid fermentation and a Meyerhof effect (22) in the former and their absence in the latter.

Discussion

Calculation shows that the ratio of the rate of fermentation to that of respiration (as measured by the CO_2 evolved) is 0.71 in normal tissue and 0.76 in tumors. This ratio was studied extensively by the early plant physiologists (23) and although varying widely in different plant tissues it had a constant value usually less than unity for a given tissue even when the rate of respiration was changed by variation in temperature, wound stimulation, variation in sugar supply to starved tissues, toxic action, and natural ageing of the tissues. In the present case the ratio is not affected by the tumor-inducing stimulus of *P. tume-*

faciens. The constancy of this ratio for a given tissue has been used (23) as an argument in favor of the Unitary Theory of the Pasteur Effect (21) since the observations support the hypothesis that many of the enzymes of respiration and fermentation are identical.

The Pasteur Effect has been defined in various ways (21) some workers preferring to regard it as the effect of oxygen in inhibiting production of the fermentation product (ethanol or lactic acid) and others regarding it as the effect of oxygen in decreasing the rate of carbohydrate catabolism of fermenting tissue.

Actually Pasteur's original observation (24) was a combination of both effects for he observed that when a suspension of fermenting yeast was oxygenated the rate of carbohydrate catabolism was decreased and alcohol production inhibited.

Both the normal tissue and tumors of the beet-root have a Pasteur Effect as far as production of the fermentation product is concerned but only the normal tissue shows a decrease in the rate of carbohydrate catabolism when air is admitted to the fermenting tissue. This existence of a Meyerhof Effect (22) together with the respiration of carbohydrate to oxalic, malic, and citric acids distinguishes the normal tissue from the tumor. It illustrates how the metabolism of tissue slices may reflect the main function of the tissue, namely storage of carbohydrate in the case of the normal tissue of the beet root. Slices of normal beet tissue possess an efficient metabolic mechanism, characterized by tissue retention of carbon rather than loss as CO_2. This is illustrated by organic acid formation in aerobic catabolism, production of lactic acid during anaerobic catabolism, and resynthesis of carbohydrate from the catabolic products when air is admitted to the fermenting tissue.

The possibility of a lactic acid fermentation occurring in tissues of the higher plants has not received much consideration although there is reason to believe such a process occurs in potato tissue (25) where anaerobic conditions result in an increase in the acidity of the tissue and subsequent release of bound CO_2. This release from carbonate is discernible as an apparent temporary stimulation of sugar catabolism (as measured by CO_2 evolved) on transference of tissue from air to nitrogen. Blackman (26) has developed an extensive theory of respiration, based on CO_2 output curves for apples under air and nitrogen, in the belief that (a) the apparent temporary stimulation, mentioned above, is due to an increase in sugar catabolism; and (b) the amount of sugar catabolized can be calculated from the CO_2 evolved by making

use of the equations for an alcoholic fermentation (nitrogen) or complete respiration of carbohydrate (oxygen). It is now evident that these assumptions are not necessarily true, since, while in *some* fermenting tissues the CO_2 evolved is a measure of the carbohydrate catabolized, in others there is a wide discrepancy. It is possible that a lactic acid fermentation is characteristic of fleshy plant storage organs since both potatoes and beet-roots fall into this category.

The fate of 75% of the sugar aerobically catabolized by tumor slices is unknown. The R.Q. is 0.92, thus suggesting that some carbohydrate may be oxidized, to a form, such as gluconic acid, not capable of reducing alkaline cupric solution. This would permit the oxygen absorbed, in excess of that required for an R.Q. of unity, to convert the maximum amount of carbohydrate to a substance not determined as reducing sugar.

The first workable scheme for the alcoholic fermentation of sugars by yeast (27) was based largely on Neuberg's studies of the effect of sulfites on the metabolism of yeast cells. He demonstrated the presence of an enzyme which decarboxylates pyruvic acid to acetaldehyde, the latter not accumulating normally because of reduction to ethanol. However, when sulfites were added to the fermentation medium, acetaldehyde and glycerol were produced from the sugar in equal proportions, the glycerol supposedly being formed by reduction of glyceraldehyde. This effect of bisulfite has been ascribed to its affinity for the carbonyl group, and hence the technique has come to be known as a "trapping" technique. The results obtained with beet tissues are not wholly in agreement with this conception since other carbonyl group reagents do not affect respiration while bisulfite will cause complete inhibition. It is more likely that the bisulfite acts by a poisoning effect on some enzyme or enzymes.

The bisulfite technique has shown that acetaldehyde is an intermediate in the formation of ethanol by ground peas (28) and it appears likely the same is true of beet tissues where bisulfite inhibits fermentation and allows accumulation of some acetaldehyde as well as glyceraldehyde. The inhibiting effect of bisulfite differs from that of fluoride in that no phosphate esters accumulate in the former case, and this probably explains why the important rôle of phosphate esters in fermentation did not become evident to Neuberg (26).

It is debatable whether results obtained with tissue slices are applicable to the original intact tissues, since the act of slicing causes a

large increase in the rate of respiration, and it is uncertain whether an abnormal type of metabolism is not induced through injury to the tissues. Although these were alive at the end of the experimental period (as indicated by plasmolytic tests) their life period ceased within a few hours thereafter. Unfortunately there are many technical difficulties in studying the metabolism of such tissues under the normal condition of growth, but experiments with tissue slices at least can be regarded as a measure of the capability of the tissues in question.

Summary

A comparative study of respiration, fermentation, and the Pasteur Effect of the normal tissue of the beet-root and of its tumor tissue induced by *P. tumefaciens* shows:

(1) The possible existence of phosphorylations in the respiration of both types of tissue, this being more marked in the case of the normal.

(2) Complete inhibition of respiration by sodium bisulfite but not by other carbonyl group reagents.

(3) Aerobic metabolism of carbohydrate to malic, oxalic, and citric acids in normal tissue and conversion of 75% of the carbohydrate to unknown substances by tumor tissue.

(4) The presence of a lactic acid fermentation and Meyerhof Effect in normal tissue which is not found in tumor, an alcoholic fermentation predominating in the latter.

(5) The presence of a well-developed Pasteur Effect in both tissues with respect to inhibition of ethanol or lactic acid formation and failure of oxygen to decrease the rate of carbohydrate catabolism of fermenting tumor slices.

(6) The accumulation of acetaldehyde and glyceraldehyde in bisulfite poisoned tissue slices.

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Studies on Plant Tumors. Part III.

Nitrogen Metabolism of Normal and Tumor Tissues of the Beet Root[1]

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Introduction

Striking differences in the chemical composition of the normal tissues of several plants and of the tumors induced on them by *Phytomonas tumefaciens* have been reported by several groups of workers (1). One of the most marked is the Kjeldahl nitrogen content which is three times as great in tumor tissue, this increase being accompanied by a corresponding decrease in the sugar content. The authors have made a study of the distribution of this tissue nitrogen and of the synthesis of protein and amides by living slices of tumors and normal tissue of tumorous beet roots.

Experimental

Beets of the variety "Extra Early Flat Egyptian" were grown and tumors induced on them by inoculation with *P. tumefaciens*, as previously described (2). The metabolism of slices of tumors and the normal tissue of tumorous beets was studied in the presence and absence of oxygen both with and without addition of ammonium sulfate to the medium. The same general technique as described previously (2) was employed, and the study included measurement of the distribution of the nitrogen in the two types of tissue.

[1] This work forms part of a thesis presented by A. C. Neish to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Holder of Fellowship from the National Research Council of Canada.

Analytical Methods

Asparagine, glutamine, and free ammonia (3) were estimated in the aqueous extracts. Non-protein nitrogen represents the total Kjeldahl nitrogen in the filtrate after the proteins have been precipitated by 4 per cent trichloroacetic acid. All other methods used were the same as those described previously (2).

Fractionation of Proteins

Water- and alkali-soluble protein fractions were obtained by grinding the fresh beet tissues under distilled water in a porcelain mortar using washed quartz sand as abrasive. The suspension was centrifuged, the clear supernatant fluid decanted off and the residue washed with water. The combined supernatant fluid and washings were mixed with an equal volume of ethanol, acidified with acetic acid and allowed to stand overnight. The precipitated water-soluble protein was removed by centrifuging, washed two or three times with 70 per cent ethanol and dried at 65°C./14 mm.

The residue from the aqueous extraction was extracted twice with 0.4 per cent NaOH at room temperature using centrifugation to separate the soluble from the insoluble material. Acidification (HCl) of the extract precipitated the alkali-soluble protein which was separated, redissolved in 0.4 per cent NaOH, reprecipitated as before, washed three times with 70 per cent alcohol and dried as above.

Results

Distribution of the Tissue Nitrogen

Tables I and II show the distribution of Kjeldahl nitrogen in normal and tumor tissues of the beet root. While the two tissues contain about the same amount of non-protein nitrogen, the tumors have a protein content over three times that of normal tissue. The greatest difference exists in the water-soluble protein nitrogen content of tumors which is six times that of the normal tissue.

Another striking difference is that tumors maintain 64% of their Kjeldahl nitrogen in the form of protein while with normal tissue this amounts to only 39%, thus reflecting the greater tendency of the tumors to synthesize proteins (1). The proportionately large amount of water-soluble protein in tumors is suggestive of a relation between rapid growth and increased solubility of the protein.

TABLE I

Distribution of Nitrogen in Beet Tissues

mg. of N per 100 g. of fresh tissue

	Tumors	Normal Tissue of Tumorous Beet
Total Kjeldahl N	436	227
Non-protein N	156	140
Protein N (by difference)	280	87
as % of total N	64%	38.5%
Water soluble protein N	96	16
as % of total protein N	34.1%	18.5%
Alkali soluble protein N	28	9
as % of total protein N	10.0%	10.4%
Residual protein N (by difference)	157	62
as % of total protein N	55.9%	71.1%

TABLE II

Nitrogen Metabolism of Beet Tissue Slices

mg. (as N) per 100 g. of fresh tissue

	Tumors			Normal tissue of tumorous beet		
	Initial Amount	Amount after 5 hrs. of Anaerobic Metabolism	Amount after 5 hrs. of Anaerobic Metabolism followed by 5 hrs. of Aerobic Metabolism	Initial Amount	Amount after 5 hrs. of Anaerobic Metabolism	Amount after 5 hrs. of Anaerobic Metabolism followed by 5 hrs. of Aerobic Metabolism
Total Kjeldahl N	441 431	436 423	422 431	223 232	223 223	213 223
Non-protein N	175 137	190 157	144 124	144 137	144 128	142 132
Protein N (by difference)	266 294	246 269	279 307	79 95	79 95	71 91
Free ammonium N	0.22 0.24	0.20 0.26	0.36 0.53	0.11 0.11	0.22 0.26	0.16 0.20
Glutamine N	1.58 1.42	0.66 0.74	0.64 0.80	1.13 1.12	0.78 0.87	1.64 1.83
Asparagine N	nil 0.14	2.47 1.96	1.00 0.87	0.27 0.43	2.10 1.83	0.60 0.04

Nitrogen Metabolism of Beet Tissue Slices

Tumor tissue slices, metabolizing anaerobically in the absence of added ammonium sulfate, show a slight breakdown of protein which is resynthesized when air is admitted (Table II). This effect is absent in normal tissues and is another indication of the more active protein metabolism of the tumor tissue. The changes in free ammonia are slight under these conditions, but there is an interesting variation in glutamine and

TABLE III

*Aerobic Metabolism of Beet Tissue Slices in the Presence of Ammonium Sulfate**

mg. per 100 g. of fresh tissue

	Tumors		Normal Tissue of Tumorous Beet	
	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism	Initial Amount	Amount after 3 hrs. of Aerobic Metabolism
CO_2 evolved		148.1 174.5		39.6 35.2
Total reducing sugar after inversion	733 742	275 275	2750 2720	2612 2569
Free ammonium ion	150 150	120 120	156 156	120 120
Ammonium from hydrolysis of glutamine	nil 6	30 30	6 7	36 36
Ammonium from hydrolysis of asparagine	nil nil	49 49	nil nil	53 45
Non-protein nitrogen	264 272	202 199	244 235	232 228
Citric acid	189 188	132 124	41 41	57 57
Malic acid	548 537	365 336	242 247	487 515

* 150 mg. of ammonium ion added per 100 g. of fresh tissue.

TABLE IV

*Anaerobic, and Subsequent Aerobic, Metabolism of Beet Tissue Slices in Presence of Ammonium Sulfate**

mg. per 100 g. of fresh tissue

	Tumors			Normal Tissue of Tumorous Beet		
	Initial Amount	Amount after 5 hrs. of Anaerobic Metabolism	Amount after 5 hrs. of Anaerobic Metabolism followed by 3 hrs. of Aerobic Metabolism	Initial amount	Amount after 5 hrs. of Anaerobic Metabolism	Amount after 5 hrs. of Anaerobic Metabolism followed by 3 hrs. of Aerobic Metabolism
CO_2 evolved		265 185	512 427		94	268
Reducing sugars	532 315	256 165	238 128	83 101	83 74	183 142
Total reducing sugars after inversion	1580 596	1490 367	665 186	4240 2158	3921 1880	3876 1925
Non-protein nitrogen	232 170	200 182	196 182	234 232	206 230	206 238
Free ammonium ion	150 150	112 124	112 124	150 150	112 124	112 120
Free ammonium from hydrolysis of glutamine	13 13	44 26	57 26	13 8	38 35	53 38
Free ammonium from hydrolysis of asparagine	nil nil	43 49	30 30	nil nil	49 26	36 12
Reactive carbonyl	11 10	16 11	15 14	7 6	10 9	11 12
Ascorbic acid	19 20	26 25	19 25	13 7	13 10	13 10
Keto acids (as pyruvic acid)	15 16	11 13	9 11	11 11	9 8	7 6
Citric acid	167 175	117 124	92 108	92 115	74	83 74
Malic acid	394 300	170 238	163 158	364 375	326 300	316 340

* 150 mg. of ammonium ion added per 100 g. of fresh tissue.

asparagine content, the former decreasing under anaerobic conditions while the latter shows a marked increase. When air is readmitted the original state tends to be re-established. This occurs with both types of tissue (Table II).

Experiments were then made to determine the effect of ammonium sulfate addition on the aerobic metabolism of beet tissue slices (Table III). Fixation of some of the added ammonium ion as glutamine and asparagine occurs with both tissues, while protein synthesis, as measured by the decrease in non-protein nitrogen, occurs in tumors but only to a slight extent in the normal tissue. Under anaerobic conditions (Table IV) synthesis of protein does not occur but ammonium ion is again fixed as glutamine and asparagine. There is considerable utilization of malic acid during fixation of the ammonia in tumor slices, citric acid also being utilized but to a lesser extent. The normal tissue synthesizes these acids under aerobic conditions just as it does in the absence of added ammonium sulfate (2). However, the ammonium sulfate actually modifies the organic acid metabolism in that keto acids and malic acid decrease under anaerobic conditions rather than increase as they do in its absence (2).

Discussion

In general the tumor tissue exhibits a greater tendency towards protein synthesis than the normal tissue as shown by the relatively greater proportion of nitrogen present in the form of protein and the utilization of non-protein nitrogen for protein synthesis under aerobic conditions. The fact that protein synthesis occurs only under aerobic conditions is in agreement with the observation of Maver, Johnston and Voegtlin (4) that a change from anaerobic to aerobic conditions in animal tumor autolyzates results in a synthesis of some protein from the degradation products.

Fife and Ferguson (5) observed that glutamine decreased, and asparagine accumulated, in the leaves of beet plants exposed to an atmosphere of 80–100% CO_2. In view of the above results (Table II) it is probable that this change was caused by lack of oxygen rather than by a specific effect of the CO_2. This may prove to be a general phenomenon since it is found in both root and leaf tissues.

According to modern theories of protein metabolism (6) malic acid can give rise to asparagine by oxidation to oxaloacetic acid, followed by amination, while glutamine can be formed similarly by oxidation of

citric acid to α-ketoglutaric acid and amination. An alternative route for asparagine formation from malic acid is by direct addition of ammonia to fumaric acid, the latter formed by dehydration of malic acid (7, 8). Thus asparagine might be formed either aerobically by the former or anaerobically by the latter method, while glutamine could be formed only aerobically. Glutamine is the amide normally present in the beet root, but it is replaced by asparagine under anaerobic conditions. This and the fact that malic acid is utilized under anaerobic conditions (Table II) follows directly from the above theories which suggest asparagine may be synthesized from malic acid under anaerobic conditions while glutamine synthesis would occur only under aerobic conditions.

In the presence of added ammonium sulfate, however, glutamine is synthesized under anaerobic conditions, citric and keto acids being simultaneously metabolized. It is possible that through dismutation reactions enough α-keto-glutaric acid can be formed from citric acid to account for the glutamine synthesized, otherwise it is necessary to postulate an anaerobic mechanism for its formation analogous to that for asparagine formation (7, 8).

Summary

A comparison of the nitrogen metabolism of normal and tumorous tissues of beet roots inoculated with *P. tumefaciens* shows:

(1) Tumor tissue has a greater tendency to synthesize proteins than the normal as evinced by its larger protein contents (both relative and absolute), and ability to synthesize protein from added ammonium sulfate.

(2) With both types of tissue, protein synthesis occurs under aerobic rather than anaerobic conditions.

(3) Glutamine is replaced by asparagine in both types under anaerobic conditions, the data suggesting its synthesis from malic acid.

(4) Ammonia nitrogen, when added to tissue slices in the form of ammonium sulfate, is partially fixed as amide nitrogen by both types of tissue.

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Studies on Plant Tumors. Part IV.

Oxidases in Normal and Tumor Beet Root Tissue

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Introduction

Previous work on the enzymatic differences between normal and tumorous tissue of beet roots has been carried out along two main lines (i) chemical analysis of both types of tissues, this showing, as a main feature, a very marked increase in formation of protein and simultaneous decrease in carbohydrate and (ii) experiments with surviving tissue which showed an increased breakdown of sugar and enhanced oxygen uptake in tumor tissue (1).

Nagy, Riker, and Peterson (2) found that extracts from galls induced on tomatoes by inoculation with *P. tumefaciens* rapidly destroyed tyrosine while stem extracts did not. Catalase, oxidase, and peroxidase activities on a wet weight basis were found to be 160, 130, and 120% higher, respectively, in galls than in normal tissues. Calculated on the basis of total nitrogen, they were 86, 73, and 57% respectively.

Klein and Ziese (3) found that catalase activity in crown gall of beets was greatly increased over that of contiguous tissue. They also found (4) in galls developed on horseradish that the increase in catalase was paralleled by an increase in peroxidase.

Neish and Hibbert (5) have shown by analytical studies that one of the major metabolic processes in tumors, produced on *Beta vulgaris* (beet) by inoculation with *Phytomonas tumefaciens* (crown gall organism), is the decrease of sucrose and increase of protein, in particular water-soluble protein.

The authors are concerned with a possible interrelationship between oxidative enzymes and protein synthesis. So far, oxidases for the following substrates have been examined: dihydroxymaleic acid, as-

corbic acid, catechol, hydroquinone, and resorcinol, and a marked difference has been observed in the two types of tissues. Other substrates are under investigation, as well as the possible rôle played by recently-isolated lignin products (6).

Experimental

Preparation of Material

Beets of the variety "Extra Early Flat Egyptian" after reaching about two to four cm. in diameter were inoculated with *Phytomonas tumefaciens*. These inoculations were made on one side of the crown only (5). The tumors, which sometimes exceeded in size the remainder of the beet root, developed on one side only, at the point of inoculation. Time for maximum growth was six to eight weeks.

Tumorous beets were harvested for experimental purposes four to ten weeks after inoculation as were also the healthy beets, planted at approximately the same time.

Manometric Measurements

Rates of respiration in air were measured by use of the Dixon and Keilin (7), as well as by the Warburg apparatus. A phosphate buffered medium (pH 7.0) was used for all substrates tested with the exception of dihydroxymaleic acid the rate of which was determined in an acetate buffer at pH 4.0 (8). The bath was kept at 28°C.

The surviving tissue technique was employed, about eight to twelve slices of fresh tissue (total weight 200–500 mg.) being used for each vessel. Each slice was approximately 1 mm. thick, 100 mm. long, and 50 mm. wide.

In a typical experiment the tissue was allowed to respire for sixty minutes, the amount of oxygen consumed being taken as unity. The substance under investigation was added and respiration allowed to proceed for an additional sixty minutes. In some experiments a period, for diffusion, of 15 minutes was allowed between time of substrate addition and commencement of determination of oxygen consumption. Control experiments showed a linear type respiration of slices of both types of tissues for at least four hours.

Parallel experiments were carried out simultaneously with healthy and tumorous tissue. The healthy tissue used was obtained from a non-infected beet rather than from contiguous normal tissue of a tumorous beet since the activity of the latter was found to be much weakened.

TABLE I

Oxygen Uptake of Normal and Tumorous Beet Root Slices

Substrate: Dihydroxymaleic Acid

Dixon-Keilin apparatus. Each vessel contained 1.0 cc. $M/5$ acetate buffer (pH 4.0), 1.5 cc. water, 0.5 cc. $M/10$ dihydroxymaleic acid (in ethanol) in side arm; 0.5 cc. ethanol only in side arm of control vessel.

mm.^{3} O_2 uptake/gram fresh tissue/hour

Trial No.	Normal Tissue	Tumor Tissue
1	616	721
2	708	886
3	418	721
4	618	734
5	686	868
6	480	782
7	542	722
8		800
9		745
Mean	581	775

TABLE II

Oxygen Uptake of Normal and Tumorous Beet Root Slices

Substrates: Ascorbic acid, Catechol

Warburg apparatus. Each vessel contained 1.5 cc. water, 1.0 cc. $M/5$ phosphate buffer (pH 7.0); 0.5 cc. $M/10$ substrate in side arm.

% Increase in oxygen uptake/gram fresh tissue/hour

Trial No.	Ascorbic Acid		Catechol	
	Normal Tissue	Tumor Tissue	Normal Tissue	Tumor Tissue
1	578	303	98	66
2	633	279	98	42
3	468	292	104	60
4	557	322	115	71
5	357	276	233	54
6	491	180	241	55
7	571	198	212	31
8	588	266	172	54
9	761	312	231	57
10	531	241	131	54
11	682	201	97	59
12	514	241	94	43
13	539	229	164	67
14	323	219	174	48
15		199	150	54
16		185	92	30
17		168	112	27
18		184	160	50
19		179		
20		195		
Mean.........	542	233	148	51

TABLE III

Oxygen Uptake of Normal and Tumorous Beet Root Slices

Substrate: Resorcinol

Warburg apparatus. Each vessel contained 1.5 cc. water, 1 cc. *M*/5 phosphate buffer (pH 7.0); 0.5 cc. *M*/10 resorcinol in side arm.

% Inhibition/gram fresh tissue/hour

Trial No.	Normal Tissue	Tumor Tissue
1	13	34
2	19	12
3	18	12
4	9	13
5	12	29
6	13	35
7	15	31
8	13	21
9	19	32
10	17	30
11	16	20
12	19	16
13	17	15
14	11	33
15	15	
16	8	
Mean....................	14	23

TABLE IV

Oxygen Uptake of Normal and Tumorous Beet Root Slices

Substrates: Resorcinol plus Catechol

Each vessel contained 1.0 cc. water, 1.0 cc. *M*/5 phosphate buffer (pH 7.0); 0.5 cc. *M*/10 resorcinol in one side arm and 0.5 cc. *M*/10 catechol in second side arm. Catechol in side arm in case of Dixon-Keilin apparatus.

% Inhibition/gram fresh tissue/hour

Trial No.	Apparatus	Normal Tissue	Tumor Tissue
1	Warburg	93.5	85.8
2	Warburg	87.5	80.3
3	Warburg	93.8	85.8
4	Warburg		78.4
5	Dixon-Keilin	95.0	85.4
6	Dixon-Keilin	96.3	
Mean.............................		93.2	83.1

Oxygen consumption was calculated as mm.^{3} per gram fresh tissue per hour.

Results on the various substrates tested are summarized in Tables I to IV.

The percentage values in Table II were obtained by the following calculation.

$$\% \text{ increase} = \frac{Q_2 - Q_1}{Q_1} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Q_1 = mm.^{3} of oxygen consumed during first hour of respiration;
Q_2 = total mm.^{3} of oxygen consumed during the entire experiment (2 hours).

The percentage inhibition values in Table III were obtained by the following calculation.

$$\% \text{ inhibition} = \left(\frac{\text{control 2nd hour}}{\text{control 1st hour}} - \frac{Q_2 - Q_1}{Q_1}\right) \times 100.$$

The values in Table IV represent the inhibition by resorcinol of increased oxygen uptake above the normal respiration due to the activation by catechol. The percentage values were obtained by the following calculations:

(*a*) *Warburg*

$$\% \text{ inhibition} = 100\left[1 - \frac{\% \text{ increase due to catechol + resorcinol}^*}{\% \text{ increase due to catechol}^*}\right]$$

* % increase due to catechol + resorcinol, and % increase due to catechol were calculated according to Equation 1.

(*b*) *Dixon-Keilin*

$$\% \text{ inhibition} = 100 \times \frac{\text{mm.}^3\ O_2 \text{ uptake by catechol + resorcinol}}{\text{mm.}^3\ O_2 \text{ uptake by catechol}}$$

Control experiments without addition of substrate were run concurrently throughout each experiment.

Discussion

While in some of the results considerable variability is noticeable, this is due solely to the physiological differences of the individual beets. Nevertheless certain conspicuous differences in their oxidases are seen to exist between crown gall and normal tissue.

Dihydroxymaleic Acid

Dihydroxymaleic acid has been postulated as a key substance in plant metabolism by Szent-Györgyi (8). One could therefore expect its activity to be increased in zones of increased growth, as in tumors. Since Theorell and Swedin (9, 10, 11) have found dihydroxymaleic acid oxidase and peroxidase to be one and the same enzyme, its rôle in plant respiratory processes is very important. While dihydroxymaleic acid activates the rate of oxygen uptake in both normal and tumor tissue, this activation is greater in the latter, 581 cmm. of oxygen being consumed by one gram of fresh healthy tissue in one hour compared to 775 cmm. for tumor tissue (Table I). Hence the activity of dihydroxymaleic acid oxidase is approximately 33% greater in tumor tissue than in normal tissue as measured on a wet weight basis. This result is in accordance with the findings of Nagy, Riker, and Peterson (2) and of Klein and Ziese (4) who measured the peroxidase activity by other methods. It also lends further support to Theorell's claim of the identity of peroxidase and dihydroxymaleic acid oxidase.

Ascorbic Acid

Since ascorbic acid is found in larger amounts in crown galls than in normal tissues (12) and since its rôle as growth factor in plants (peas) has been demonstrated by von Hausen (13), the action of ascorbic acid oxidase in both types of beet root tissue was examined.

Ascorbic acid (Table II) increases the oxygen uptake in both types of tissues. It is a much more powerful donator in normal tissue (542% increase) than in tumor (233% increase); there is less ascorbic acid oxidase in tumors, thus possibly accounting thereby, in part, for their higher ascorbic acid content.

Catechol

Catechol is a strong donator for both normal and tumorous beet root tissues (Table II). The average increase in oxygen uptake was 148% in normal tissue and 51% in tumor. Catechol oxidase is therefore more prevalent in healthy tissue.

Catechol oxidase was not isolated from the beet, but the comparative strengths of the oxidase in both types of tissue were studied *in vivo*.

Hydroquinone

To date it has not been possible to obtain conclusive results with hydroquinone. However, in several cases it appeared to be a donator for both types, being more powerful in tumor tissue.

Resorcinol

In contradistinction to catechol and hydroquinone, resorcinol, the third dihydroxybenzene isomer, is an inhibitor for both types of tissue, the inhibition being greater in tumor (23%) than in normal tissue (14%) (14). These findings are in agreement with those of Richter (15) that catechol oxidase isolated from different sources is inhibited by resorcinol.

Since resorcinol inhibits respiration in both types, different oxidative systems were subsequently examined with regard to their susceptibility to inhibition by resorcinol. Dihydroxymaleic acid oxidase and ascorbic acid oxidase were not inhibited by it.

There is an indication on the other hand that catechol oxidase is inhibited due to the fact that the increased oxygen uptake, observed with catechol alone, is reduced again upon addition of resorcinol. This inhibition amounts to approximately 93% in the case of normal tissue and approximately 83% in tumor tissue, *i.e.*, 10% greater in normal tissue.

Catechol oxidase is about three times as powerful in normal as in tumor tissue. Also it is seen that resorcinol inhibits oxygen uptake about 10% more in tumor tissue than in normal, indicating that the decreased oxygen uptake in tumorous tissue caused by resorcinol cannot be accounted for by catechol oxidase inhibition alone.

A further indication that peroxidase (dihydroxymaleic acid oxidase) is not involved in the resorcinol inhibition was obtained in experiments on the interaction, *in vitro*, of hydrogen peroxide and resorcinol (16). These results showed that resorcinol binds hydrogen peroxide sufficiently to prevent its detection by the customary procedures.

The effect of resorcinol on catalase activity has not yet been investigated by the authors.

Summary

Influence of dihydroxymaleic acid, ascorbic acid, catechol, hydroquinone, and resorcinol on the oxygen consumption of normal and

tumorous beet tissue was examined. An increased oxygen uptake was observed in each case with the exception of resorcinol. This latter substance inhibits catechol oxidase—both in normal and tumorous tissue. Resorcinol inhibition is not fully explained by its action on catechol oxidase. Neither dihydroxymaleic acid oxidase nor ascorbase is inhibited by resorcinol. The increased ascorbic acid content of tumors is explained in part by their lower ascorbase content.

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The Formation of Creatine from Ammonium Carbonate and Sarcosine in Vitro

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Introduction

Beard and Pizzolato (1) suggested that urea and glycine could react in the body of the rat and man to form creatine and creatinine. Fisher and Wilhelmi (2) and Bloch and Schoenheimer (3) offered several theoretical criticisms of this theory to which we have replied (4). Later we observed that urea and glycine could react *in vitro* to form glycocyamine and that urea and sarcosine would form creatinine under similar conditions (5). These observations were controlled by use of the specific creatinine enzyme of Miller and Dubos (6). There was a summation of effects on creatine and creatinine excretion in the rat and man when urea and glycine were injected in the former and ingested by the latter as compared to the effect of glycine alone (7). Similar results were later obtained using sarcosine in place of glycine (8). There was a summation in the extra watt minute output in various individuals and students when urea and glycine were ingested in wine as compared to the watt minute output in the same individuals with identical amounts of glycine in wine alone (9, 10).

In recent years it has become apparent that the nitrogenous waste products of the urine may serve a useful function in the body. Urea can replace fairly large amounts of protein in meeting the nitrogenous requirements of certain types of animal, and urea, ammonia, creatinine, and uric acid may all be transformed into creatine (11). It is, therefore, reasonable to believe that some of the precursors of urea might also be transformed into creatine. We have shown this with both ornithine and arginine (1).

In a preliminary study it was observed that the solution obtained after incubating ammonium carbonate and sarcosine at 60°C. after acid

hydrolysis gave the Jaffe reaction with alkaline picrate. In another study it was found that the addition of muscle tissue alone of all the other tissues studied, increased the intensity of the Jaffe reaction. At the end of 10 days incubation the total creatinine was 6 mg. per 100 cc. of solution as compared to 2.2 mg. total creatinine in the control muscle flask. These observations were controlled by the use of the specific creatinine enzyme of Miller and Dubos (6). In the present study further work on this reaction was completed, the results of which are reported below.

Experimental

The first three 200 cc. Erlenmeyer flasks contained ammonium carbonate and sarcosine alone; the next three contained muscle tissue alone; the next three boiled muscle tissue with ammonium carbonate and sarcosine, and the last nine contained fresh muscle tissue with ammonium carbonate and sarcosine. 10 g. of ammonium carbonate and 10 g. of sarcosine were dissolved in 1500 cc. of phosphate buffer to a final pH of 7.9, and 100 cc. of this solution were used in all flasks except those containing the muscle tissue alone. All solutions were incubated under toluene from 5 to 25 days at 40°C. The pH of each solution was determined at 5 day intervals with the Beckmann pH meter. Aliquots of the filtered solutions of each flask were used to determine the total and preformed creatinine using alkaline picrate, and the Fisher electrophotometer was used for measuring color intensities. The specific creatinine enzyme of Miller and Dubos was used when deemed necessary.

Results and Discussion

Traces of creatine were formed in the ammonium carbonate + sarcosine flasks (Table I). These were so small, however, that they might have been due to experimental error. Fresh muscle tissue, or boiled muscle tissue with ammonium carbonate + sarcosine, gave similar increases in the Jaffe reaction. The addition of fresh muscle tissue to the ammonium carbonate + sarcosine flasks, however, gave increases in creatine from 5 to 25 days later. Study of several of these solutions showed that, in every case, the color of the Jaffe reaction disappeared after treatment with the specific creatinine enzyme so that it is certain that we are measuring creatine, as creatinine, in the solutions.

At the end of 5 days incubation in the presence of fresh muscle tissue the average formation of creatine was 211 per cent greater than was found in the control muscle tissue solutions, either fresh or boiled muscle tissue, etc. This indicates that fresh muscle tissue contains an enzyme which hastens the formation of creatine from ammonium carbonate and

sarcosine (*cf.* 5, 12). For want of a better term we will name this enzyme *createse* for the present. At the end of 15 days incubation all values for creatine in control and experimental flasks were about the same. The

TABLE I

Effect of Incubation of Ammonium Carbonate with Sarcosine, with and without Muscle Tissue, upon Creatine-Creatinine Formation

(mg./100 cc. solution)

Tissues added to Solutions	Days of Incubation at 40°C.														
	5			10			15			20			25		
	PC[1]	C as C[2]	pH	PC	C as C	pH	PC	C as C	pH	PC	C as C	pH	PC	C as C	pH
Control (1 g. $(NH_4)_2CO_3$ + 1 g. Sarcosine/100 cc.)	0.0	0.0	7.3	0.0	0.0	7.4	0.0	0.0	7.8	0.0	0.7	8.0	0.4	1.0	7.9
	0.5	0.7	7.4	0.0	0.0	7.3	0.0	0.0	7.9	0.0	0.2	7.9	0.0	0.3	7.9
	0.0	0.2	7.6	0.0	0.0	7.5	0.0	0.0	7.3	0.0	0.0	8.0	0.0	0.5	8.0
Muscle (4 g. /100 cc.)	0.0	5.6	7.0	0.0	4.6	7.0	0.0	19.0	7.1	1.0	13.0	7.1	0.0	8.0	6.9
	0.0	5.0	6.9	0.0	4.7	6.9	0.0	18.5	7.0	1.5	14.0	6.8	0.0	9.2	7.0
	0.0	5.1	7.0	0.0	4.5	7.0	0.0	19.2	7.1	1.1	13.1	7.1	0.0	10.0	7.1
Boiled Muscle (4 g./100 cc. + 1g. $(NH_4)_2$-CO_3 + 1 g. Sarcosine)	0.0	4.8	7.1	0.0	4.6	6.9	0.0	19.0	6.9	1.1	13.5	6.9	0.0	11.0	7.0
	0.0	5.2	6.9	0.0	5.1	7.0	0.0	18.5	7.0	1.0	12.8	7.0	0.0	10.2	7.1
	0.0	5.1	7.0	0.0	4.7	7.0	0.0	17.0	7.1	1.4	13.3	7.1	0.0	11.3	7.0
Muscle (4 g. + 1 g. $(NH_4)_2$-CO_3 + 1 g. Sarcosine-/100 cc.)	0.0	4.0	7.4	0.0	17.0*	7.6	0.0	19.8*	7.2	3.5	30.5	7.3	4.5	23.5*	8.1
	0.0	4.0	7.4	0.0	7.0	7.4	0.0	19.0	7.7	0.4	18.6	8.0	0.4	17.6	8.0
	0.0	_17.5_*	7.7	0.0	17.6	7.7	0.0	19.0	7.6	4.5	30.5	8.0	3.0	33.6*	8.1
	0.0	_22.5_	7.6	0.0	17.6	7.6	0.0	19.0*	7.8	4.5	35.5*	8.0	3.0	30.0	8.1
	0.0	_20.0_*	7.7	0.0	19.0*	7.7	0.0	24.5	7.7	4.5	35.5	8.0	3.0	33.0	7.9
	0.0	_20.0_	7.6	0.0	15.0	7.7	0.0	22.0	8.0	4.0	30.0	8.0	3.0	33.0*	8.1
	0.0	_20.0_	7.6	0.0	16.0	7.6	0.0	19.0	8.0	5.5	28.5	8.0	3.0	30.0	8.0
	0.0	_21.5_	7.7	0.0	14.6*	7.1	0.0	24.5	7.2	1.0	18.2	7.3	0.8	14.2	7.9
	0.0	_19.0_	7.7	0.0	17.0	7.6	0.0	19.0	7.6	4.0	30.0	8.0	1.4	18.6	7.9

[1] PC = preformed creatinine.

[2] C as C = creatine "as creatinine."

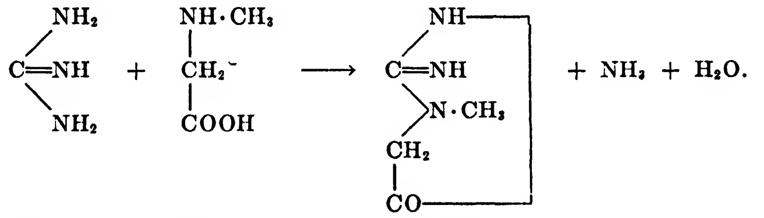
* Color of the Jaffe reaction destroyed by the Miller and Dubos specific creatinine enzyme.

disappearance later in the control flasks of some creatine offers additional evidence that muscle tissue contains an enzyme which we have named *creatine oxidase* which destroys creatine (13). This proof here is, how-

ever, not conclusive since there was also a disappearance of creatine in the boiled muscle flasks. In any case it is apparent that the newly formed creatine did not disappear from the experimental flasks. The small amount of creatinine appearing at 20 days incubation may represent that formed by the slow dehydration of creatine or an independent formation of creatinine from ammonium carbonate and sarcosine under these conditions.

Beard and Espenan (5) showed the catalytic effect of ammonia on creatine formation *in vitro* confirming the earlier observations of Strecker (14) in this connection (*cf.* Hunter, 15). Beard and Pizzolato (1) observed that the amino group of the amino acid was necessary for creatine formation in the rat. Bloch and Schoenheimer (16, 17) stated that ammonia containing N^{15} could be transferred to creatine containing N^{15}. These observations show the central position occupied by ammonia in creatine formation *in vivo* and *in vitro*.

Horbaczewski (18) and Paulmann (19) heated guanidine carbonate with sarcosine from 140 to 160°C. and obtained creatinine by acidifying the product with HCl and evaporating the syrup to dryness. These syntheses established the structure of both creatine and creatinine as they are known today.



Guanidine Sarcosine Creatinine

Our results show that ammonium carbonate can replace guanidine carbonate in this reaction so that this synthesis is analogous to that of the earlier workers mentioned above.

Controls using muscle with sarcosine were not run since, under these conditions, only a very small amount of extra creatine is formed (5). The reader may not be convinced that sarcosine forms creatine. The following facts are offered in this connection, (a) sarcosine is demethylated to glycine in the body; (b) glycine is a precursor of creatine; and (c) sarcosine forms more creatine in the rat and man than does an

equivalent amount of glycine (11). Since the control muscle creatine and that formed by the addition of ammonium carbonate and sarcosine with muscle tissue were about the same at the end of 15 days incubation one may also get the impression that these substances did not increase the creatine values here. The data, however, at 5 and 20 days incubation makes it evident that, at these intervals, more creatine was formed in the presence of ammonium carbonate with sarcosine than in the control muscle flasks. This would indicate that this was due to an enzymatic formation of creatine here in the presence of muscle tissue. There is also a difference of 0.5 pH in the control and ammonium carbonate + sarcosine flasks but it is not believed that this had anything to do with the change.

Summary

Creatine formation from ammonium carbonate and sarcosine *in vitro* was studied. Rat muscle tissue contains an enzyme, *createse*, which caused 211 per cent more creatine formation from these substances than was formed in the control muscle tissue flasks. Use of the Miller and Dubos specific creatinine enzyme showed that true creatine was the substance responsible for the color of the Jaffe reaction with alkaline picrate under these conditions. Ammonium carbonate can replace guanidine carbonate in the Horbaczewski-Paulmann theory of creatine formation from sarcosine and guanidine carbonate.

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The Influence of the Rate of Protein Metabolism upon Creatine-Creatinine Transformation and Excretion in the Rat

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Introduction

Until recently the transformation of creatine into creatinine was an accepted fact and probably the most convincing evidence of it was published by Bloch and Schoenheimer (1) using N^{15} in creatine and isolating creatinine with N^{15} from the urine. On the other hand there are those who deny this change, and both sides of the question have been discussed (2). All of our results point to the creatinine → creatine transformation in the rat and man, and we have shown the presence of a *creatinine hydrase,* but no *creatine anhydrase,* in different rat tissues (3).

An interesting transformation of creatine into creatinine in the dog was reported by Bollman (4). The creatinine excretion was decreased when a low protein diet was fed, and the addition of creatine did not increase it. If, however, 100 g. of casein were added to the diet, in 2 weeks the creatinine excretion began to rise and remained high even after the administration of creatine was discontinued. According to Bollman this proves the transformation of creatine into creatinine and shows the effect of the rate of protein metabolism on the change.

We were interested in studying the creatine → creatinine transformation in the rat in relation to the level of protein metabolism. In this paper the terms "creatine and creatinine" refer to creatine and creatinine that originate from a normal level of protein metabolism as contrasted to "injected or administered" creatine and creatinine. We have shown that the body metabolizes these two types of creatine and creatinine in a different manner, and it is of much importance to keep this fact in mind (2). The terms creatine and creatinine, therefore, have little or no meaning unless the above distinction is made.

Our results, which show that the conclusions of Bollman for the dog do not hold for the rat, lead to the following deductions, (1) the rate of protein metabolism greatly influences creatine and creatinine excretion, (2) injected creatine with Amigen, with one exception, instead of increasing creatinine excretion, causes creatinine retention instead, (3) injected creatinine causes an increase in creatine excretion which is independent of the rate of protein metabolism, and (4) the injection of glycine or arginine to the rats fed on the low protein diet does not form creatine as would be the case if the animals were fed on a normal protein diet.

Experimental

Ten adult rats were divided into two groups of 5 each. The urine from each was collected in 3-day periods and was preserved with 5 cc. of 3 per cent HCl. Purina Dog Chow (hereinafter called checkers) containing 20 per cent protein was fed to all groups except one which received the checkers containing 5 per cent protein, the 15 per cent protein being replaced at the Purina Mills in St. Louis by an equal amount of carbohydrate. Food intakes were measured as accurately as possible. Amigen, a 10 per cent casein digest of Mead, Johnson and Company, was administered in place of the drinking water. Total and preformed creatinine were determined as usual using alkaline picrate. The Fisher electrophotometer was used for measuring color intensities.

Results and Discussion

The results obtained are given in Charts 1 and 2. In the first periods of both groups the usual amounts of creatine and creatinine were excreted. Of the 9 checkers given daily, each weighing from 2 to 5 g., several were always left in the cage the next morning. In this period both groups ingested about 20 g. of the 20 per cent checkers daily (4 g. protein). In the second period of both groups only 3 checkers were allowed daily. (19 g. = 19 × 5% = 0.95 g. protein) It will be noticed that a marked drop in both creatine and creatinine excretion occurred in both groups. In further studies to be discussed below both creatine and creatinine practically disappeared from the urine when the 5 per cent checkers were fed. These results show conclusively the effect of the rate of protein metabolism upon creatine and creatinine excretion.

In the third period 6 checkers were given daily resulting in an average food intake of 21 g. (4.2 g. protein). An immediate increase of over 600 per cent in creatine excretion occurred with practically no increase in creatinine excretion. This observation also shows the independence of the rate of protein metabolism on the excretion of creatinine in these two

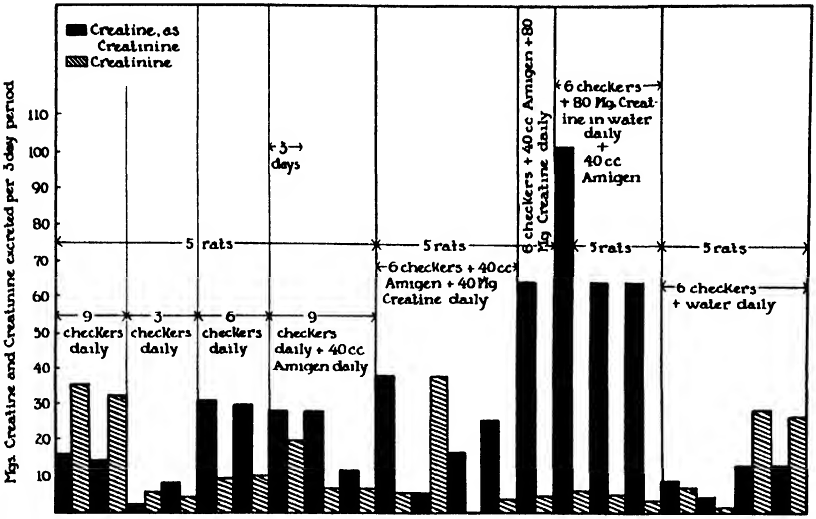

CHART 1

Effect of Amigen Ingestion upon Transformation of Administered Creatine to Creatinine

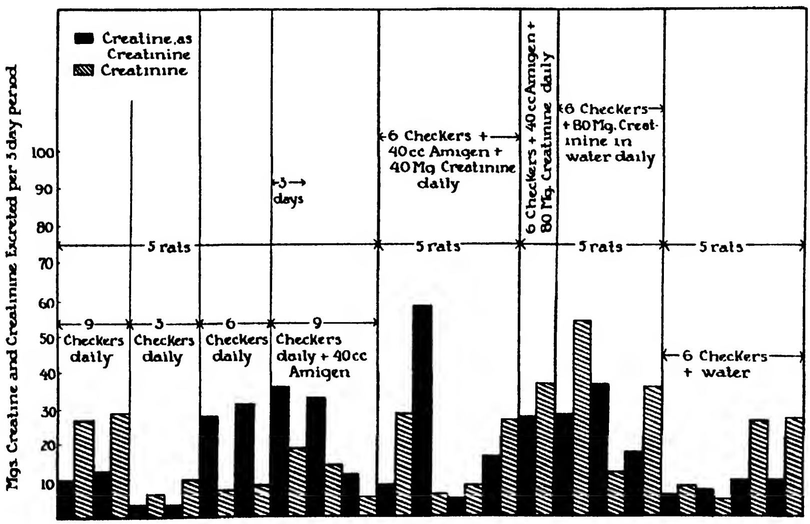

CHART 2

Effect of Amigen Ingestion upon Transformation of Administered Creatinine to Creatine

studies. In the fourth period 9 checkers were again given and 40 cc. of Amigen given in place of the drinking water. This was quantitatively consumed in all but four instances in which a total of 8 cc. were refused. An average of 19 g. of the checkers was consumed (3.8 g. protein) with the 4 g. of casein digest, or about 23 g. in all. The excretion of creatine was not influenced over that of the preceding period with only a slight increase in creatinine excretion in one period. The 18 mg. of creatinine excreted here was about 10 or 15 mg. less than that excreted in the first control periods. In the fifth period the injection of 40 mg. creatine (Chart 1) or a similar amount of creatinine (Chart 2), each with 40 cc. of Amigen solution, were given. From this point we will discuss the results of each group separately.

In Chart 1 it is seen that there was only a slight increase in creatine excretion (over the average of the five preceding periods) during the first 3 days of the fifth period and no increase in creatinine excretion. In the next 3-day period the creatine excretion was greatly lowered and the creatinine excretion markedly increased. This would indicate that the level of protein metabolism influences the transformation of injected creatine into creatinine (Bollman) or, more likely, that there was a retention of creatinine during the preceding periods. Evidence in favor of the latter view is the fact that in two succeeding periods creatinine almost disappeared from the urine, while, in the dog the creatinine excretion would remain high under these conditions (4). In the next period, 80 mg. of creatine were injected with the 40 cc. of Amigen. Very large increases in creatine excretion occurred together with a *retention* of creatinine (*cf.* periods 5 and 6 with 3 and 4). We have often observed this phenomenon in the rat (2). During these three 4-day periods 960 mg. of creatine were injected, and only 282, or 29 per cent, were excreted. A retention of 70 per cent of the creatine caused a retention of about 20 mg. of creatinine daily as compared to the control creatinine excretion in the first periods. This would seem to be conclusive proof that *injected* creatine is not transformed into body creatinine in the rat. In the next period with the elimination of the creatine and Amigen the creatine and creatinine excretions soon returned to their control levels.

Now let us return to Chart 2 during the fifth period. The injection of creatinine in the animals fed the 20 per cent checkers (3.83 g. protein) with Amigen resulted in an increase in creatine excretion of 50 mg. above the control level, or 555 per cent. This was due to a transformation of injected creatinine into creatine which was independent of the rate of

protein metabolism. In all 160 mg. of creatinine were injected, and 68 mg. were excreted together with 86 mg. of creatine. This transformation of about 95 per cent of retained creatinine into creatine might be considered quantitative in this instance. In the sixth period, the 181 mg. of creatinine retained was transformed into 109 mg. of creatine, which represents a 60 per cent transformation. In our previous study, when 40 and 75 mg. of creatinine were injected once, the retained creatinine which was transformed into creatine was 153 and 83 per cent, respectively (5).

Beard, *et al.* (6). fed 5 grams each of urea and glycine to two adults. An excretion of 35.5 g. of extra creatine and creatinine was observed, (theoretical 36.5 g.). In rats the creatine excretion was directly proportional to the amounts of glycine and urea fed. Under these conditions, if body creatine was transformed into creatinine, there should have been a retention of some of the creatine but such was not the case. This is still further evidence that creatine is not transformed into creatinine in the rat. At the present time there is no method of determining whether body creatine is transformed into body creatinine.

The above results indicate that the rate of protein metabolism influences the metabolism and excretion of body creatine and creatinine with, however, little influence on the metabolism and excretion of *injected* creatine and creatinine. Bollman (4) believes that the rate of protein metabolism influences the latter change. If so this may be a species difference between the rat and dog. Since he did not feed 100 g. of casein without the creatine to his dogs we are of the opinion that he was obtaining an increased rate of creatinine formation and excretion from the casein rather than from the creatine. This is further shown by the continued increase in creatinine excretion when glucose replaced casein in the diet.

During the second periods shown in Charts 1 and 2 the feeding of 3 checkers daily (0.95 g. protein) caused a big drop in both creatine and creatinine excretion. This was due either to a lack of calories or protein. To test this point the 20 per cent checkers (3.73 g. protein) were fed with the usual amount of creatine and creatinine excretion (Chart 3). At the beginning of the fifth 3-day period the animals were then fed on the 5 per cent checkers (0.94 g. protein). After 12 days almost all of the creatine and creatinine had disappeared from the urine. At this time the animals were again fed on the 20 per cent checkers (3.85 g. protein). The creatine excretion went even higher in the fourth period than in the control periods on the same diet while the excretion of creatinine soon

reached its control level. Since the food intakes of both types of the checkers was again about the same it is seen that the lowered creatine and creatinine excretions could not have been due to a lack of calories.

The study was then repeated with the same animals with similar results. When either 200 mg. of glycine or arginine were injected into animals receiving the 5 per cent checkers (0.89 g. protein) there was only a very slight increase in creatine and creatinine excretion, and these were much below the normal output of these substances on the 20 per cent checkers (4.01 g. protein) (Chart 3). It is, therefore, seen that these amounts of the amino acids will not increase creatine formation on the 5

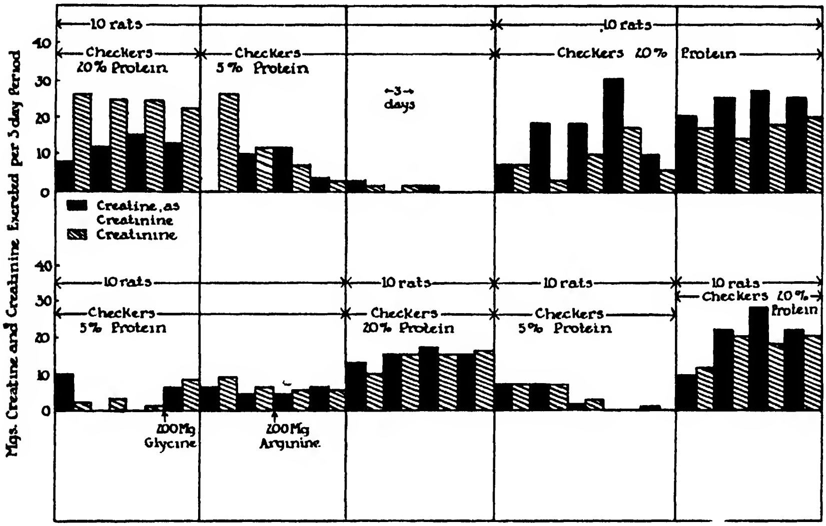

Chart 3

Effect of Feeding 5 and 20 Percent Checkers on Creatine-Creatinine Excretion

per cent protein diet, while it is well known that they will do so on the 20 per cent protein diet (2). We have often stated that if one feeds a low protein diet to animals or patients the administered amino acids will be used first for protein synthesis with little or no creatine formation.

Summary

Purina Dog Checkers containing either 20 or 5 per cent protein were fed to adult rats, with and without injections of creatine and creatinine, to determine the rate of creatine and creatinine excretion in the urine. The following results were obtained:

1. Creatine and creatinine almost disappeared from the urine of the animals fed on the 5 per cent checkers. This was due to a lowered protein intake rather than to a lack of calories. Injection of glycine or arginine did not form much creatine under these conditions.

2. An immediate increase of over 600 per cent in creatine excretion with no increase in creatinine excretion occurred in two studies, but an increase in creatinine occurred in a third study when the animals were fed on the 20 per cent checkers. This shows the effect of the rate of protein metabolism upon creatine formation and excretion in animals fed on a normal protein diet.

3. The rate of protein metabolism on the other hand does not influence the excretion of injected creatine and creatinine, and no evidence was obtained to show that this type of creatine was transformed into creatinine, since the latter was retained under these conditions.

4. Injected creatinine caused over 500 per cent increase in creatine excretion which was again independent of the rate of protein metabolism.

5. This is further evidence that the body metabolizes and excretes administered or injected creatine and creatinine differently from what it does in the case of body creatine and creatinine, *i.e.* that formed during a normal rate of protein metabolism.

We wish to thank Dr. W. M. Cox, Jr., of Mead Johnson and Company, for the samples of Amigen used, and Dr. H. J. Smith of the Purina Mills for the preparation of the 5 per cent checkers.

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Some Components of the Seed Coats of the Common Bean, Phaseolus vulgaris, and Their Relation to Water Retention

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Introduction

Considerable work has been presented on the permeability of seed coats of the common bean to water and salt solutions (1). Little work, however, has been reported on the chemical composition of the seed coats. Schulze and Pfenninger (2) reported hemicelluloses to be a large constituent of the bean coats, the content of hemicelluloses reaching a maximum in the ripe bean.

The closely related cell wall pectic substances are among the most hydrophilic compounds present in plants. The building units of these pectic substances are for the most part uronic acids. A knowledge of the uronic acid content, or more properly the polyuronide content of the seed coats, might be a step toward an explanation of water absorption. Pentosans, one of the most important components of the many hemicelluloses, are believed to be closely related to polyuronides; and their determination has been confused with the determination of uronides. Proteins are also well known to be functional in water absorption (3).

The confusion in the determinations of pentosans and polyuronides is due to the fact that both yield furfural on treatment with strong HCl and heat. Two methods have commonly been used to distinguish between these two groups of compounds. One based on the observation of Lefèvre and Tollens (4), who in 1907 pointed out that glucuronic acid liberated CO_2 when heated with strong acid, and the second method, also developed by Tollens (5), based on the color produced when glucuronic acid was heated with naphthoresorcinol in the presence of HCl. Several improvements in the CO_2 method have been published, in particular, those by Dickson, Otterson, and Link (6), and Phillips, Goss, and

Browne (7). The specificity of the naphthoresorcinol method was challenged a number of years ago by Mandel and Neuberg (8). Many of the interferring substances occur in plants; therefore, its use in the analysis of plant materials would be subject to criticism.

Experimental

The seed coats from a mixture of strains of mature beans grown in Michigan were first loosened from the beans by placing them in distilled water for fifteen minutes. By use of rubber rollers, the seed coats were removed from the beans. Rapid drying with warm air from an air blast made final separation of the heavy bean from the seed coats relatively easy. The seed coats were air-dried, ground, and passed through a 20-mesh sieve. The ground seed coats were divided into four lots. Lot I was left untreated; Lot II was treated with 1 per cent acetic acid for ten hours at 60° and pH of 2.8; Lot III was treated with 1 per cent sodium bicarbonate solution for 10 hours at 60° and pH of 7.8; while Lot IV was treated with 0.8 per cent sodium hydroxide for 10 hours at 60° and pH of 11.5. All these lots were washed seven times with distilled water and then air dried.

Moisture determinations were made by the oven-dry method in which samples were dried to constant weight at 100–110° C., and all analyses reported are on the oven-dry basis.

Uronic acids were determined by the method of Dickson, Otterson, and Link (6). The method was first checked by analysis of Eastman's α-d-galacturonic acid. Duplicate analyses gave a recovery of 100.3 per cent and 100.1 per cent galacturonic acid. The uronic acid content of the seed coats is expressed as uronic acid anhydride or polyuronide. Since one molecule of CO_2 is liberated from each polyuronide residue, the percentage of CO_2 times 4 gives the percentage of polyuronide. The procedure for the determination of pentosans was essentially that published in the Methods of Analysis of the Association of Official Agricultural Chemists (9); steam distillation, however, was used for the recovery of the furfural. This method involves the assumption that the pentosans consist of equal amounts of araban and xylan. Hockett, *et al.* (10) have shown that even if d-lyxose and d-ribose were the furfural yielding substances, in place of arabinose and xylose, the error introduced would not be larger than that associated as a consequence of the difference between arabinose and xylose. "True pentosans" were calculated from the "total pentosans" by correction for the furfural liberated from the polyuronides.

By "total pentosans" is meant the pento sans equivalent to the total furfural. Each residue weight of polyuronide yields one molecular weight of furfural. However, Norris and Resch (11) found only about 42 per cent of the furfural expected when uronic acids were determined in the presence of common plant materials. By reference to Kröber's tables (9) the pentosans equivalent to this corrected value of furfural, given by the polyuronide, if deducted from the "total pentosans" gives the so-called "true pentosans." For example, the percentage of polyuronide in the untreated seed coats is 19.39 per cent; the furfural equivalent 10.56 per cent; the corrected value 4.44 per cent, which in turn is equivalent, according

to Kröber's tables, to 7.61 per cent pentosans. This latter figure deducted from the "total pentosans" would give the so-called "true pentosans."

Total protein was determined by a semi-micro Kjeldahl method and the protein calculated by use of the factor 6.25 times the nitrogen.

The results of these analyses are reported in Table I. Each value submitted is the average of three or four closely checking determinations.

TABLE I

Composition of the Seed Coats of the Common Bean, Phaseolus vulgaris

Treatment	(All values given are percentages)				
	Moisture	Protein	Poly-uronide	"Total Pen-tosans"	"True Pen-tosans"
		dry weight	*dry weight*	*dry weight*	*dry weight*
None	8.07	5.07	19.39	29.03	21.42
1 per cent CH_3COOH	8.94	5.11	19.36	29.35	21.76
1 per cent $NaHCO_3$	11.78	4.61	19.24	29.20	21.64
0.8 per cent NaOH	4.55	2.99	16.24	25.78	19.12

Discussion

The total combined content of polyuronide and "true pentosans" of the untreated seed coats is in the neighborhood of 40%. Schulze and Pfenninger (2) found a maximum of 48.65% of hemicellulose in the ripe bean coats. The uronide content of the bean coats compares favorably with many of the higher yielding hemicellulose preparations from various plants discussed by Norman (12).

A comparison of the moisture content of the four lots of seed coats gives an indication of the importance of the uronides and pentosans in the water retention of the dried coats. Treatment with 1 per cent acetic acid gave seed coats of slightly increased moisture content, when air-dried, as compared with the air-dried, untreated seed coats. The sodium bicarbonate treated samples showed the highest moisture content, 11.7%, while the sodium hydroxide treated samples gave the lowest moisture content, namely 4.55%. The sodium hydroxide samples also showed greatest loss of uronide, "true pentosans," and protein—as calculated from the nitrogen content. The sodium bicarbonate treatment gave a small definite loss of protein and no appreciable loss of uronide or "true pentosan." If the water retention was correlated with

the nitrogen, that is, the protein content, one would expect a decrease in the water retention of these bicarbonate treated samples; the opposite condition, however, was observed.

Summary

1. The polyuronide and the "true pentosans" content of the seed coats of the common bean, Phaseolus vulgaris, are about 19% and 21% respectively of their dry weight.

2. From nitrogen determinations, the protein is about 5% of the dry weight of the seed coats.

3. Neither treatment with 1 per cent acetic acid nor 1 per cent sodium bicarbonate alters the polyuronide or "true pentosans" content of the seed coats; however, 0.8 per cent sodium hydroxide treatment lowers the content of both polyuronide and "true pentosans."

4. Protein is slightly lowered by sodium bicarbonate treatment and noticeably decreased by treatment with sodium hydroxide.

5. There is indication that polyuronides and "true pentosans" are involved in water retention in the dried seed coats.

The authors wish to thank Dean R. C. Huston, Professor of Organic Chemistry, for helpful suggestions in this work.

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The Dispersion of Keratins

II. Studies on the Dispersion of Keratins by Reduction in Neutral Solutions of Protein Denaturants

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Introduction

In a study of the dispersion of certain keratins in alkaline solutions of sodium sulfide (1) it was found possible to suppress protein degradation to such an extent that the dispersed proteins could be recovered nearly quantitatively by acid precipitation. Data reported in the present paper show that some keratins may be readily dispersed at neutral reaction, without recourse to high temperatures, by cleavage of the disulfide bonds in the presence of protein denaturants. In the preparation of stable keratin dispersions and in the isolation therefrom of a protein in which it is desirable to retain, to as high a degree as possible, the characteristics of the original keratin, the advantages of the elimination of alkali are self-evident. Although dispersion of keratins in neutral media has been reported by other investigators (2–5), the methods used have involved high temperatures over long periods of time, oxidizing agents, and other factors known to produce extensive breakdown of proteins.

As previously pointed out by the authors (1) in discussing the dispersion of keratins by reduction in alkaline solutions, it may be concluded from work reported by Goddard and Michaelis (6) and by Harris and co-workers (7) that reduction of the disulfide groups and alkaline cleavage of secondary linkages are mutually independent reactions, both of which are necessary for dispersion of keratins. The function of the alkali may be regarded, then, as that of a dispersing agent for the reduced keratin. Considering the dispersion of keratins from this

standpoint, it seemed possible that dispersion might be effected by reduction of the keratins in solutions of protein-dispersing agents other than alkali.

Several substances in aqueous solution are known to denature native proteins and to increase the solubility of both native and denatured proteins. These include urea, formamide, acetamide, ammonium thiocyanate, guanidine, surface-active agents such as synthetic detergents and bile salts, and various other compounds. Several of these were investigated and were found to disperse keratins in conjunction with the chemical action of sodium bisulfite, monothioglycol, or neutralized thioglycolic acid. Experiments were performed first on the keratins of chicken feathers, cattle hoof, hog hair, and wool. Since a wide variation in dispersibility between these keratins was observed, the studies were extended to include other keratins. It will be shown that dispersibility in neutral solutions varies greatly between different dispersing and reducing agents as well as between different keratins.

Experimental

The keratins investigated were prepared from the following materials: chicken body feathers, cattle hoof, hog hair, wool, white duck down, cattle horn, human hair, egg-shell membrane, snake skin, and tortoise scutes. The first four of these were handled as previously described (1), the chicken feathers being ground in a Wiley mill equipped with a 2-mm. screen before they wĕre extracted with benzene. The cattle-horn keratin was prepared by the procedure described for the preparation of cattle-hoof keratin. The duck-feather keratin was prepared in the manner described for chicken-feather keratin, except that it was not ground. Human hair from barber-shop clippings was separated from extraneous material and was then washed several times with cold water, air-dried, and extracted with benzene; the resulting product was finally washed with water and air-dried. Ovokeratin was prepared by the method of Calvery (8) (with the exception that benzene extraction was substituted for the alcohol and ether washings) from frozen egg-shell waste from a commercial egg-breaking plant.[1] The snake skin and tortoise scutes[2] were each washed repeatedly with water, air-dried,

[1] The egg-shell waste used in these studies was supplied through the courtesy of the Washington Cooperative Egg and Poultry Association, Seattle, Washington.

[2] We are grateful to Dr. Joseph R. Slevin, of the California Academy of Sciences, for furnishing us with the snake skins and tortoise scutes; they were from the Banded Rattlesnake, *Crotalus horridus horridus* (Linné), and the Desert Tortoise, *Gopherus agassizii* (Cooper).

and extracted with benzene. They were air-dried, again washed with water and air-dried, and were finally ground in a small Wiley mill equipped with a 20-mesh screen. All benzene extractions were carried out in Soxhlet extractors for at least 15 hours.

Analytical data on the keratin preparations are presented in Table I. Total nitrogen was determined by the Kjeldahl method, ash by dry ignition at about 500°C., and moisture by drying in an oven at 105°C. for 15 hours. Total sulfur was determined by an alkaline permanganate fusion at 600°C. (9).[3] Cystine and cysteine were determined by a modification of Vassel's methods (10).[4]

TABLE I

Analytical Data on Keratin Preparations

Keratin	Moisture	Ash[1]	Nitrogen[2]	Sulfur[2]	Cystine[2]	Cysteine[2,3]
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Chicken feather.....	8.8	0.73	16.2	2.9	7.2	0.4
Duck feather	9.3	0.36	16.2	2.9	9.2	0.4
Tortoise scutes	8.8	0.57	16.2	1.4	4.2	0.7
Snake skin..........	9.5	2.31	15.9	2.2	4.8	0.6
Cattle hoof..	9.4	1.24	16.9	2.1	5.5	0.4
Wool......... .	9.9	0.79	16.7	3.7	11.4	0.4
Cattle horn.........	9.7	0.44	16.9	3.9	10.5	1.6
Hog hair............	8.5	0.38	16.9	3.8	12.5	0.8
Human hair.........	8.7	0.72	16.4	4.7	16.6	0.8
Ovokeratin..........	11.1	0.01	15.7	4.7	10.9	1.1

[1] Corrected for moisture.
[2] Calculated on basis of moisture-free, ash-free material.
[3] As half-cystine.

The dispersibilities of the keratins were investigated in solutions containing a disulfide-splitting agent and a dispersing agent. The disulfide-splitting agents included in this study were thioglycol (11), thioglycolic acid, and sodium bisulfite; the dispersing agents were urea, guanidine, ammonium thiocyanate, formamide, acetamide, thiourea, and a synthetic detergent of the sodium alkyl sulfate type.[5] Each solution was made up to the desired concentration, the pH being adjusted to 7.0 ($\pm$ 0.2) by addition of NaOH solution or HCl. Two and one-

[3] Nitrogen, moisture, and ash determinations were carried out by A. Bevenue, and sulfur analyses were performed by E. F. Potter, both of this Laboratory.

[4] A report on the modification of these methods is being prepared for publication.

[5] Duponol C, manufactured by E. I. duPont de Nemours and Company, Inc.

half grams of keratin was treated with 35.0 ml. of solution in a stoppered 50-ml. Erlenmeyer flask suspended in a constant-temperature bath at 40°C. for 18 hours, during the early part of which the flask was shaken at frequent intervals. At the end of this period the dispersion mixture was cooled to approximately room temperature and the pH was noted. Two grams of Hyflo filter aid was incorporated in the mixture, which was then filtered through a Büchner funnel. The residue was washed with several portions (about 100 ml. in all) of a solution of the corresponding dispersing agent (without the reducing agent) in half the concentration in which it had been used for dispersion. This preliminary washing was performed because, in some cases, direct addition of water caused precipitation of the dispersed protein in the funnel. The residue was washed finally with at least 250 ml. of water and was dried in an oven at 103°C. overnight. It was then exposed to the air at room temperature for 24 hours before it was weighed; moisture determinations made on a few residues treated in this way showed the moisture content to be approximately that of the original keratin (8 to 10 per cent). Extent of dispersion of the keratin was calculated from the net weight of the residue.

Results obtained with thioglycol as the reducing agent in the presence of various protein-dispersing substances are presented in Table II. When a solution of 0.5 *M* thioglycol and 10 per cent Duponol was used as the dispersing medium, the weights of the undispersed residues were in some cases greater than the weights of the keratin samples. This retention of a portion of the Duponol in the keratin residues was considered to be a result of the formation of a complex between the detergent and the undispersed residues; complex formation has been reported between similar detergents and non-keratin proteins (12, 13). It was found that the Duponol could be removed from the protein-detergent complex by extraction with 60 per cent aqueous acetone at room temperature.[6] After three successive extractions with 50-ml. portions of 60 per cent acetone the residues were dried as described above and were then weighed. As shown in the last column of Table II, the extent of dispersion as calculated from the final weight agreed quite well in each case with the value obtained by calculation from the nitrogen contents of the filtered dispersion and of the original keratin.

Samples of the keratins were dispersed in 10.0 *M* solutions of urea, using 0.5 *M* thioglycol and 0.5 *M* thioglycolic acid (neutralized with

[6] Suggested by Dr. H. P. Lundgren of this Laboratory.

NaOH) as reducing agents. In a third series 0.3 M $NaHSO_3$ was employed as the agent for producing the necessary cleavage of the disulfide bonds in the keratins. This concentration of bisulfite had been found to be optimal for dispersion of wool and feather keratins in urea solutions under the conditions used here. Extent of dispersion for each of the keratins in 10.0 M urea is shown in Table III.

In the experiments described above, no attempt was made to maintain the solutions at constant pH during the dispersion period by means of buffers. A phosphate-citrate buffer was found to inhibit dispersion

TABLE II

Dispersibilities of Keratins in Different Dispersing Agents upon Reduction by 0.5 M Thioglycol

2.5 g. of keratin was treated for 18 hours at 40° C. with 35 ml. of solution at pH 7

Keratin	Guanidine (8.1 M)	NH_4-CNS (9.0 M)	Formamide (10.0-M)	Acetamide (10.0-M)	Thiourea (1.2 M)	Duponol[1] (10%)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Chicken feather	84	82	66	59	10	79, 80
Duck feather	83	80	41	36	6	51, 53
Tortoise scutes	64	52				10, 8
Snake skin	55	44				26, 30
Cattle hoof	74	56	7	6	5	58, 64
Wool	61	36	4	6	4	44, 50
Cattle horn	36	27	3	5	4	14, 12
Hog hair	56	26	2	2	2	4, 3
Human hair	50	11	0	0	2	2, 1
Ovokeratin	8	6	4	3	15	2, 4

[1] First values calculated from weights of air-dried residues after extraction of detergent; second values calculated from results of nitrogen analyses (see text).

of wool in urea-$NaHSO_3$ solution to some extent. Over two-thirds of the dispersions reported had final pH values between 6.0 and 7.5 before filtration; the lowest final pH observed was 4.7 (hog hair-thiourea-thioglycol) and the highest was 7.8 (ovokeratin-urea-thioglycol).

The dispersibilities of each keratin in alkaline solutions of reducing agents were determined for comparison with those in the neutral solutions. Sodium sulfide (0.1 M), sodium thioglycolate (0.5 M), and thioglycol (0.5 M) solutions were prepared, the pH of the thioglycolate and thioglycol solutions being adjusted to 12.7; the procedure then

followed was that described for the neutral dispersions except that the residues were washed directly with water. The results are presented in Table IV.

TABLE III

Dispersibilities of Keratins in 10 M Urea upon Reduction by Different Disulfide-Splitting Agents

2.5 g. of keratin was treated for 18 hours at 40° C. with 35 ml. of solution at pH 7

Keratin	Thioglycol (0.5 *M*)	Thioglycolic acid (0.5 *M*)	$NaHSO_3$ (0.3 *M*)
	per cent	*per cent*	*per cent*
Chicken feather	80	73	80
Duck feather	78	78	82
Tortoise scutes	52		
Snake skin	44		
Cattle hoof	30	23	39
Wool	27	29	52
Cattle horn	22	22	36
Hog hair	12	6	52
Human hair	4	5	26
Ovokeratin	2	2	5

TABLE IV

Dispersibilities of Keratins in Alkaline Solutions of Reducing Agents

2.5 g. of keratin was treated for 18 hours at 40° C. with 35 ml. of solution at pH 12.7

Keratin	Thioglycol (0.5 *M*)	Sodium thioglycolate (0.5 *M*)	Sodium sulfide (0.1 *M*)
	per cent	*per cent*	*per cent*
Chicken feather	86	84	85
Duck feather	86	83	85
Tortoise scutes	89	86	68
Snake skin	55	48	40
Cattle hoof	76	52	79
Wool	83	76	73
Cattle horn	76	50	64
Hog hair	85	71	71
Human hair	81	68	52
Ovokeratin	7	1	6

The values presented in this paper as percentages of dispersion obtain only under the conditions described and are affected by variations in such factors as temperature and time of digestion. The extent

of dispersion is also dependent on the size of the keratin particles. Hoof-keratin filings were dispersed in urea-thioglycol to the extent of 30 per cent, whereas, by the same treatment, 53 per cent dispersion was obtained after the material had been ground in a Wiley mill equipped with a 20-mesh screen. In the case of feathers (which present a larger surface area), such grinding was found to have no effect on the extent of dispersion.

By the method used in these experiments the values obtained were usually reproducible to within 4 per cent dispersion although occasional variations of as much as 6 per cent were encountered. A few experiments on hoof keratin indicated that consistent differences in dispersibility—greater than the limit of experimental error—may exist between different lots of the same keratin; however, feather and horn keratins prepared from different lots of material showed no such variation. The results presented in Tables II and III show that dispersibility varies significantly between keratins in the same dispersing medium and between dispersing and reducing agents for any one keratin.

In addition to the dispersing agents described above, other substances known to have a denaturing and solvent effect on proteins are effective in dispersing keratins in a reducing solution. For example, when adjusted to neutrality, 35.0 ml. each of sodium salicylate (4.0 *M*), urethane (7.5 *M*), and phenol (70%) solutions in 0.5 *M* thioglycol dispersed 81, 72, and 73 per cent, respectively, of 2.5 g. of chicken-feather keratin during 18 hours at 40°C.

No significant dispersion occurred when chicken-feather keratin was treated, under the conditions of the above experiments, with each of the dispersing agents in the absence of a reducing agent, or with thioglycol, neutral thioglycolate, or sodium bisulfite alone. The largest values were 7 and 5 per cent dispersion, obtained respectively with sodium salicylate and urea. These control experiments were performed only on feather keratin since this keratin was, in general, the most readily dispersed in solutions containing dispersing agent and reducing agent together. No evidence was observed for the formation of a complex between Duponol and unreduced feather keratin.

The neutral keratin dispersions were clear liquids of low viscosity, similar in appearance to dispersions obtained by alkaline reduction. Dispersions of the non-pigmented keratins were usually blue-green in color, whereas those of the pigmented keratins, such as feathers and hair, were dark brown, although the ratio of pigment in the dispersion

to that in the residue varied somewhat with the dispersing agent. Preliminary experiments have shown that the dispersed protein may be precipitated by salting out (*e.g.*, with $MgSO_4$ or $(NH_4)_2SO_4$), acidification, addition of protein precipitants such as trichloroacetic acid, or by removal of the dispersing agent by dialysis. In certain cases the protein may be precipitated to some extent by addition of alkali to about pH 8, and in other cases merely by dilution of the dispersion with water.

Discussion

The foregoing experiments present the only means, so far as we are aware, whereby a keratin can be dispersed at neutral pH and at as low a temperature as 40°C. The most drastic alteration which the protein undergoes during such dispersion is the cleavage of disulfide bonds. Since hydrolytic action is minimized under these conditions, the polypeptide chains of the reduced keratin in the resulting dispersions are in all probability more nearly identical with those of the original keratin than are those of the "kerateine" described by Goddard and Michaelis (14). It is hoped that this method of dispersion, employing neutral reducing agents, may provide a new approach in the elucidation of the chemistry of keratins.

A noteworthy feature of these results is the wide variation between keratins in susceptibility to dispersion in neutral reducing solutions. Of the keratins investigated in this work the feather keratins and ovokeratin are least similar to the other keratins in this respect. The feather keratins were *most* readily dispersed in all the combinations of reagents used (with the exception of thiourea-thioglycol solution), the upper limit of dispersion being 80 to 85 per cent—a value which was not increased by decreasing the ratio of keratin to dispersing solution or by increasing the concentration of the reagents. This was also the upper limit of dispersibility shown by any of the keratins in the alkaline reducing media. Ovokeratin was consistently the *least* dispersed of the keratins by any of the reagents with the exception of thiourea-thioglycol solution, which, of the reagents used, was least effective in dispersing the other keratins. To determine whether the acid treatment involved in the preparation of the ovokeratin might have had an effect on its dispersibility, a preparation was made in which the acid treatment was replaced by manual separation of the membrane from the egg-shell. The dispersibility of the ovokeratin thus prepared was the same as that

of the preparation reported here. The resistance of ovokeratin to dispersion in alkaline reducing solutions is even more striking than that in neutral solutions.

Preliminary experiments have indicated that the extent of reaction between the disulfide groups of wool keratin and sodium bisulfite (0.3 *M* $NaHSO_3$, pH 7, 18 hours, 40°C.) is greater in the presence than in the absence of several of the dispersing agents described here. No correlation was apparent, however, between extent of cleavage of the disulfide groups and extent of dispersion of the keratin. A similar increase in the availability of the disulfide groups of wool has been reported by Ramsden (15), who observed that treatment of wool with alkaline saturated urea solutions increased the extent of reduction by cyanide as shown by the nitroprusside reaction. An increase in the availability of disulfide groups has also been observed to occur upon denaturation of non-keratin proteins (16–18). It seems likely, therefore, that dispersion of keratins is the result of the combined reactions of "denaturation" and reduction. However, it is not yet possible to account for the unusual susceptibility of certain keratins (*e.g.*, feather keratin) and resistance of others (*e.g.*, ovokeratin) to dispersion.

Summary

Keratins can be dispersed at neutral reaction by cleavage of their disulfide bonds, either by reduction with sulfhydryl compounds or by the action of bisulfite, in the presence of any of a number of substances (urea, guanidine, a synthetic detergent, and others) which act upon non-keratin proteins as denaturants. The dispersibility of one keratin may differ from that of another in a particular combination of dispersing and disulfide-splitting agents and may vary with different combinations of these agents. Of the keratins investigated, feather keratin is most readily dispersed in neutral solutions, and ovokeratin is unique in its resistance to dispersion not only in neutral solutions but also in alkaline reducing solutions.

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The Distribution of Lipids in Animal Tissues[1]

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Introduction

Although newer concepts of biochemistry envisioning a dynamic state of body constituents have replaced the classical picture of a fixed system, the structural components of the organism retain a relatively constant pattern through delicately balanced cycles of closely linked chemical reactions (1). Among the structural components in this basic pattern, which differs widely among the various tissues depending on their functional significance in the organism, proteins have been attributed predominance. However, the lipids which are essential constituents of cells and cell membranes are equally important structural materials.

It has been generally accepted that the lipids occurring in the animal organism may be divided into two categories (a) the reserve or depot fat, or *element variable* and (b) the essential cellular components, or *element constant* (2). Phospholipids have usually been considered to comprise the major portion of the latter category and neutral fat or triglyceride the former. It has become apparent, however, that the phospholipids may fall into two general types, depending on the particular function they perform. Part of the phospholipid in tissues may be of the metabolic type and part non-metabolic—the latter portion representing the constitutive or structural components of cells and membranes (3). Regardless of definition both types appear to be essential to normal cell function and whether the term *element constant* covers both types of phospholipid or only the non-metabolic portion is still indefinite.

[1] Preliminary reports of these data were given before the Division of Biological Chemistry of the American Chemical Society at the 104th Meeting, Buffalo, N. Y., Sept. 7–11, 1942; and at the Annual Meeting of the Michigan Academy of Arts and Science, Ann Arbor, March 13, 1942.

Those tissues and organs which perform the greatest variety of physiological activities contain the highest concentrations of lipids (4, 5, 6). Moreover, in those tissues where activity fluctuates from time to time, parallel variations in lipid content occur. Furthermore, it has been demonstrated that artificial enhancement of activity, such as experimental exercise of muscle tissue through several generations, produces not only muscular hypertrophy but also higher concentrations of phospholipid and cholesterol in the muscle (7).

The quantitative aspects, therefore, of the relationship of lipid constituents to the functional activity of cells appears to be well established. However, the distribution of the various lipid components among the different tissues of the body and their significance as structural constituents has not been clearly defined. And, further, the particular functions of the individual lipids and their relation to the activities of the various organs remain to be determined.

This report is concerned with comparisons of the patterns of the lipid constituents of beef organs and muscles, of muscles of other animal species, and of avian and reptilian eggs.

Experimental

Each sample of tissue analyzed was a composite from a number of animals, the number varying with the tissue and the source. When two composite samples were analyzed, as in the case of the beef organs, they were prepared at different times. This sampling procedure was necessary to provide the material required for the numerous analytical determinations. The amounts of 10 amino acids in the whole protein of these composite samples are reported in another publication (8).

Each composite was dried from the frozen state under high vacuum (9) and powdered in a ball mill. Samples of the powdered material were extracted with hot ethanol, then with ethyl ether. The final extracts, combined, represented approximately a 3:1 mixture of alcohol-ether and were analyzed for total phosphorus, choline, sphingomyelin, total and free cholesterol, galactose, and acetone-soluble glycerol. *Total phospholipid* is the lipid phosphorus times 23.54. Since lecithin and sphingomyelin each contain one molecule of choline for every molecule of phosphorus, and cephalin contains no choline, the ratio between the molecular equivalents of choline to the molecular equivalents of phosphorus, multiplied by the total phospholipid, represents the amount of *choline phospholipid*. *Cephalin* is total phospholipid minus choline phospholipid.

Lecithin is total choline phospholipid minus sphingomyelin. *Cholesterol esters* is the difference between total and free cholesterol multiplied by 1.69. *Cerebrosides* is galactose times 4.55. *Neutral fat* is acetone-soluble glycerol multiplied by 9.62. The complete details of all the methods used have been published (10).

TABLE I

Lipid Composition of Beef Organs

Per cent of dry weight

	Phospholipid	Cerebroside	Free cholesterol	Cholesterol esters	Neutral fat	Total lipid†	Essential* lipid
Brain I	24.27	11.55	10.54	0.22	2.84	49.42	46.58
II	28.48	12.47	9.45	0.27	3.10	53.77	50.67
Liver I	13.75	0.00	0.49	0.63	5.62	20.49	14.87
II	18.69	0.00	0.38	0.43	5.99	25.49	19.50
Kidney I	9.06	0.56	1.44	0.27	5.23	16.56	11.33
II	11.57	0.86	1.43	0.41	3.66	17.93	14.27
Heart I	9.83	1.99	0.35	0.24	4.97	17.38	12.41
II	9.82	2.00	0.33	0.22	3.14	15.51	12.37
Lung I	9.37	0.43	1.33	1.05	2.42	14.60	12.18
II	10.20	0.44	1.35	0.68	2.39	15.06	12.67
Thymus I	6.72	0.40	0.58	0.69	2.08	10.47	8.39
II	6.70	0.45	0.30	1.01	21.90	30.36	8.46
Intestine	6.93	0.32	0.76	0.73	2.89	11.63	8.74
Stomach	3.10	0.39	0.64	0.25	3.60	7.98	4.38

† Total lipid is the sum of phospholipid, cerebrosides, free cholesterol, cholesterol esters, and neutral fat.

* "Essential" lipid is used to designate total lipid minus neutral fat and is comprised of those lipids considered to be "physiological lipids". Other terms such as non-storage fat, element constant, tissue lipid, and structural lipid have been used to designate these lipid components.

Results

The lipid composition of various beef organs is presented in Table I. Inasmuch as the neutral fat or triglyceride is considered to be storage or reserve fat and has been estimated directly by determination of the acetone-soluble glycerol, this particular lipid fraction has been subtracted from the total lipid content and the remaining lipid material, composed of the cholesterol fractions, cerebroside and phospholipid, is

considered as essential cellular lipid material and has been designated "essential lipid."

From the results in Table I, the various organs of beef range from a high for brain, with nearly 50 per cent of essential lipid, to a low for stomach, with less than 5 per cent. This range of essential lipid content is a reflection of phospholipid concentration, since this particular lipid fraction is the largest component of the essential lipids. The results confirm those of Bloor on the relation of phospholipid concentration to the physiological activity of various tissues (11), for those tissues with the greatest variety of physiological activities, such as brain and liver, had the highest concentration of phospholipid. The essential lipid was found to parallel the phospholipid content, which demonstrates the advantages of a separate determination of the variable lipid fraction, the triglyceride. For example, the total lipid content of Thymus I was 10.47 per cent of the dry tissue, of which neutral fat comprised 2.08 per cent. In contrast, Thymus II had a total lipid content of 30.36 per cent, of which neutral fat comprised 21.90 per cent. Yet the amounts of essential lipid were similar in the two samples, 8.39 and 8.46 per cent respectively.

With the exception of the brain, which contained approximately 10 per cent of free cholesterol on the dry basis, relatively small amounts of cholesterol were found in the beef organs. Kidney and lung had between 1 and 2 per cent free cholesterol and the remaining tissues studied contained less than 1 per cent. Cholesterol esters are a small but apparently a significant lipid fraction of most of the tissues. They occurred in lower concentration than free cholesterol in all tissues except liver and thymus.

Cerebrosides have hitherto been undifferentiated and have been included with the neutral fat fraction in most studies of lipid distribution. From our results, it appears that this particular lipid material occurs in significant amounts in all the organs except liver. Brain, which has long been considered the main site of cerebroside in the body, contains approximately 12 per cent on the dry basis. Among the other organs, the heart is outstanding in cerebroside concentration, containing cerebroside amounting to approximately 2 per cent of the dry substance in comparison with less than 1 per cent in the other organs studied.

The lipid distribution of the voluntary muscles of various animal species is given in Table II. The importance of differentiating the

storage and *non-storage* fatty material is again illustrated. Skeletal muscle of beef showed a total lipid content of 14.21 per cent for sample I and 9.77 per cent for sample II. This difference was due to the different amounts of triglyceride present, since sample I contained 9.97 per cent neutral fat and sample II, 5.17 per cent, making the essential lipid approximately equal in the two samples, 4.24 and 4.60 per cent, respectively. Pork muscle had the highest total lipid content (22.40 per cent) of all the muscles studied, but next to the lowest content of essential lipid, since a large part of the total lipid was neutral fat.

TABLE II

Lipid Composition of Muscle Tissue and Egg

Per cent of dry weight

	Phospholipid	Cerebroside	Free cholesterol	Cholesterol esters	Neutral fat	Total lipid	Essential lipid
Muscle:							
Frog... ..	7.14	1.76	0.18	0.08	1.72	10.88	9.16
Turtle.	5.25	0.91	0.26	0.11	10.89	17.42	6.53
Veal...... ..	5.04	0.62	0.16	0.16	5.86	11.84	5.98
Lamb........	4.74	1.95	0.13	0.18	4.19	11.19	7.00
Salmon.. .	4.39	3.96	0.11	0.08	9.76	18.30	8.54
Chicken (dark) .	4.36	1.26	0.28	0.12	6.65	12.67	6.02
Codfish... ...	4.28	2.64	0.22	0.13	2.20	9.47	7.27
Shrimp. .	3.89	1.22	0.70	0.07	2.24	8.12	5.88
Beef I..	3.08	0.94	0.19	0.03	9.97	14.21	4.24
II..	3.39	0.95	0.16	0.10	5.17	9.77	4.60
Pork....	3.06	1.17	0.14	0.06	17.97	22.40	4.43
Chicken (light)..	2.72	1.99	0.17	0.08	1.99	6.95	4.96
Egg:							
Chicken.	13.73	1.35	1.69	0.35	33.15	50.27	17.12
Turtle...	6.94	0.00	0.76	0.31	27.22	35.23	8.01

The essential lipid in muscle of the different species tends to conform with the concentrations of the phospholipid, but not as closely as in the organ tissues of beef. The phospholipid concentration, as in the organ tissues, appears to be related to the physiological activity of the different muscles, again confirming Bloor's results (12). For example, as Bloor noted, the more active dark muscle of the chicken has a higher phospholipid (also essential lipid) concentration than the less active light muscle. Likewise, the skeletal muscle of the calf (veal) has a higher concentration than that of the adult (beef).

The cholesterol content of the various muscles, with the exception of shrimp, is quite low, particularly when compared to that of the organs (Table I). Cholesterol esters occur in such small quantities in most of the muscles that it is doubtful if much significance can be attached to the presence of this lipid fraction.

It is notable that the various types of muscle (smooth, cardiac, and skeletal) here studied present a cholesterol picture in agreement with that pointed out by Bloor (6). From Table I, beef intestine and stomach (essentially smooth muscle) show the highest free cholesterol content when compared to beef heart, which is intermediate, and beef skeletal muscle (Table II) which is lowest in free cholesterol content. Although Bloor noted the distinction on the basis of total cholesterol, it holds also for free cholesterol.

A striking and unexpected finding was the uniformly high concentrations of cerebroside among the skeletal muscles of the various species. Comparison of the cerebroside contents of the three conventional types of muscle indicates that the differences may be as significant as those noted for the cholesterol content. Cardiac muscle has the highest content, approximately 2 per cent, and smooth muscle (stomach and intestine) the lowest, averaging 0.36 per cent (Table I) with skeletal muscle intermediate, approximately 1 per cent (Table II).

A comparison of the lipid composition of chicken and turtle eggs (Table II) shows that approximately 30 per cent of the dried, whole egg of either species is neutral fat. This is not unexpected when the energy demands of the developing embryo are considered. Concentrations of all lipids are consistently greater in chicken than in turtle egg. No cerebroside could be detected in the turtle egg.

The percentage distribution of the various lipid fractions in the essential lipid of all the tissues studied is shown in Figure 1. It is readily evident that the phospholipid comprises the largest part of the physiological or essential lipid in all the tissues. Of the other components, cholesterol is greater in the organ tissues, with the exception of brain, and cerebroside in the skeletal muscles.

The partition of the phospholipid in the beef organs is presented in Table III. There appears to be little if any relation between the concentrations of total and the individual phospholipids in the various tissues. Outside of the brain, which contains approximately 5 per cent of the dry weight as sphingomyelin, lung, kidney, and intestine have a notably high content of this particular phospholipid; over 1 per cent of

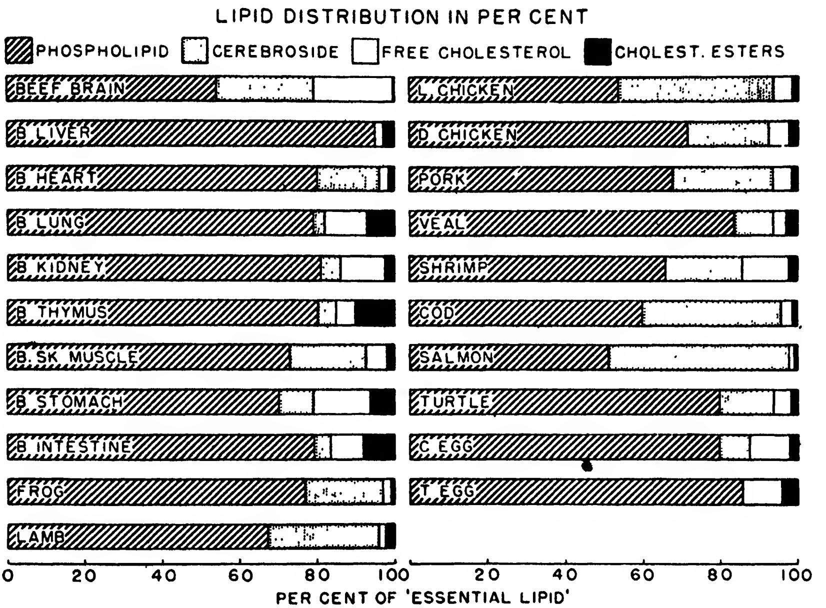

FIG. 1

TABLE III

Phospholipid Composition of Beef Organs

Per cent of dry weight

	Total phospholipid	Cephalin	Choline-phospholipids: Total	Lecithin	Sphingomyelin
Brain I	24.27	12.57	11.70	6.65	5.05
II	28.48	16.14	12.34	7.46	4.88
Liver I	13.75	4.91	8.84	8.04	0.80
II	18.69	8.28	10.41	9.69	0.72
Kidney I	9.06	2.05	7.01	5.45	1.56
II	11.57	4.00	7.57	5.80	1.77
Heart I	9.83	5.34	4.49	3.97	0.52
II	9.82	5.34	4.48	3.95	0.53
Lung I	9.37	3.71	5.66	3.34	2.32
II	10.20	4.10	6.10	3.87	2.23
Thymus I	6.72	1.96	4.76	4.09	0.67
II	6.70	3.48	3.22	2.48	0.74
Intestine	6.93	1.86	5.07	3.81	1.26
Stomach	3.10	0.88	2.22	1.60	0.62

the dry substance of kidney and intestine and over 2 per cent of the lung tissue.

The composition of the phospholipids in the voluntary muscles of various species, and in chicken and turtle eggs, is given in Table IV. The sphingomyelin content of the various muscles is strikingly low, less than 0.5 per cent of the dry weight, in comparison to that found for the various organs of beef (Table III). No sphingomyelin could be detected in the turtle and salmon muscles, nor in the light muscle of chicken. Comparison of the sphingomyelin contents of the three conventional types of beef muscles shows that the smooth muscle is highest, intestine 1.26 per cent, and stomach 0.62 per cent, cardiac contains an average of 0.53 per cent (Table III), and skeletal muscle is lowest with an average of 0.20 per cent (Table IV).

A graphic presentation of the phospholipid partition in terms of total phospholipid is given in Figure 2. Except for brain, heart, lung, frog muscle, and the dark muscle of chicken, lecithin is the largest phospholipid component in the various tissues studied. Lung, brain, intestine, stomach, and kidney are outstanding in that they contain a high proportion of the total phospholipid as sphingomyelin. In agreement with the findings of Hunter (13), who studied cat tissues, the lung of beef contains a larger percentage of the total phospholipid as sphingomyelin than does brain, although the phospholipid content of brain is nearly three times that of lung. The skeletal muscles, as well as cardiac muscles, appear to contain a relatively low proportion of the phospholipid in the form of sphingomyelin. The smooth muscles, in contrast, (in agreement with Hunter's results on cat intestine) contain a high proportion of the phospholipid as sphingomyelin.

With the exception of sphingomyelin, the concentration of the individual phospholipids of chicken egg, similar to the total, is approximately twice that in turtle egg. Despite the fact that chicken egg contains twice as much phospholipid as turtle egg, the proportions of cephalin and lecithin in the two egg types is similar. Approximately one fourth of the total phospholipid is cephalin and three fourths lecithin. The proportion of the total phospholipid as sphingomyelin in the two types of egg is very small although it may be significant that this particular phospholipid is twice as concentrated in the phospholipid of turtle egg as in chicken egg.

TABLE IV

Phospholipid Composition of Muscle Tissue and Egg

Per cent of dry weight

	Total phospholipid	Cephalin	Choline-phospholipids Total	Lecithin	Sphingomyelin
Muscle:					
Frog	7.14	4.16	2.98	2.79	0.19
Turtle	5.25	2.27	2.98	2.98	0.00
Veal	5.04	2.15	2.89	2.70	0.19
Lamb	4.74	1.90	2.84	2.55	0.29
Salmon	4.39	1.86	2.53	2.53	0.00
Chicken (dark)	4.36	2.39	1.97	1.70	0.27
Codfish	4.28	0.49	3.79	3.31	0.48
Shrimp	3.89	1.03	2.86	2.63	0.23
Beef I	3.08	1.12	1.96	1.72	0.24
II	3.39	1.27	2.12	1.96	0.16
Pork	3.06	1.25	1.81	1.69	0.12
Chicken (light)	2.72	0.81	1.91	1.91	0.00
Egg:					
Chicken	13.73	3.44	10.29	9.95	0.34
Turtle	6.94	1.85	5.09	4.84	0.25

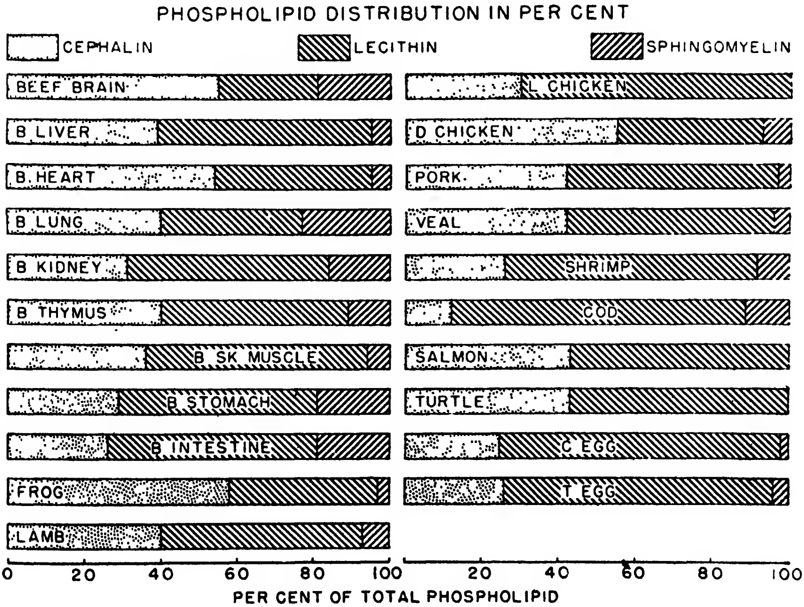

Fig. 2

Discussion

The importance to life of the chemical composition of cells and tissues seems obvious. Whether composition is of primary or secondary importance to arrangement, organization, and structure appears to be of small consequence in view of the dynamic rather than the static state of body constituents (14). Among the components of protoplasm, the lipids, as structural elements, appear to be of equal importance to that of the proteins.

As has been pointed out, the essential lipid content of a tissue is "an expression or a measure of the extent and variety of the physiological activities of that tissue" (4). Besides the essential lipid content being higher in those tissues in which the extent and variety of physiological activities are greater, the pattern of the lipid components comprising the essential lipid is different for tissues with diverse functions. In contrast, the partial pattern of the amino acids of the total tissue protein (8) indicates much greater uniformity among proteins in tissues with widely differing functions.

The phospholipids comprise the largest fraction of the essential lipid. Cerebroside is the next largest component of the essential lipid in skeletal and cardiac muscle with cholesterol as a minor constituent. On the other hand, in the smooth muscle (intestine and stomach) and the other organs, the position of these two lipid fractions is reversed. Likewise, the phospholipid distribution indicates that sphingomyelin is of greater relative importance in the soft organs of the body than in skeletal muscles.

In those tissues such as muscles, in which one form of physiological activity (energy transformation) is predominant, there is present in the cells ten to twenty times as much protein as lipid. In brain, liver, and other organs, in which variety of reactions other than oxidation appear more significant in the total physiological activity, lipid occurs in proportions of from one sixth to equivalent quantities of protein.

Parallel to the complexity of physiological processes occurring in a tissue and the ratio between protein and lipid structural materials (15) is the physical quality of a tissue. Muscles are chemico-dynamic machines whose primary function is the transformation of chemical energy into work and heat. The predominant structural material, protein, supplies the requisite physical toughness to the muscle tissue. In brain and nerve tissue where processes other than energy transformation appear relatively more significant, the protein structural material

is diluted to such an extent with lipid that the tissue has little or no physical resistance.

One point which cannot be overlooked in a study of the lipid composition of tissues was emphasized by Bloor (6)—the problem of how much of the lipid material present in a tissue is derived from nerves or neural mechanisms. It seems obvious that a certain amount must so originate depending on the nerve supply of various types of tissues. Lacking knowledge, however, of the composition of the nervous structures in tissues, it is impossible to estimate accurately the neurolipid content of the essential lipid. It has been thought that certain lipid components such as sphingomyelin or cerebroside which have been considered to be primarily of neural origin might be used as the basis for such an estimate. No sphingomyelin was found in light muscle of chicken, turtle, and salmon muscle. Furthermore, in the lung sphingomyelin occurs in greater concentration in the total phospholipid (23.3 per cent) than in brain (18.8 per cent). Likewise, liver did not contain cerebroside, and we must therefore assume either that no significant amount of neural tissue is present in this organ or that any that is present is without cerebroside in its composition. Furthermore in certain muscle tissues (lamb, salmon, codfish, pork, and light chicken) cerebroside occurs in greater proportion to the essential lipid than in brain.

Despite the fact that the necessary analytical information is still unavailable to determine the amount of lipid material of neural origin present in various tissues, the indications from histological and chemical examinations are that the major portion of the essential lipid occurring in most tissues does not arise from nervous structures present, but is an essential part of the structural and functional mechanisms of the individual cells.

The importance of the various lipid fractions to the structural and functional mechanisms of the individual cells has received added impetus in view of recent findings on the composition of tissue lipids. Halliday, *et al.* (16) have shown that cerebroside may occur in the body (at least in a pathological condition) in the form of glycolipid (glucose-containing) instead of the usual galactolipid (galactose-containing). Folch (17) has shown that all cephalin in the body may not be of the ethanolamine type (*phosphatidyl-ethanolamine*), but may be in part *phosphatidyl serine*, and possibly *inositol phosphatide*. Finally, MacLachlan, *et al.* (18) in studies with the fasting mouse have shown that whereas the *a-lecithin* and *b-cephalin* fractions of the liver phospholipids may undergo a profound change, the *b*-lecithin and *a*-cephalin fractions remain constant, and

the latter may therefore represent the true "element constant" of the tissue lipids.

Summary

The lipid [phospholipid (cephalin, lecithin, and sphingomyelin) free and combined cholesterol, cerebroside, and neutral fat] distribution in beef organs and muscles, in the muscles of other warm- and cold-blooded species, and in avian and reptilian eggs, was determined. The advantages of the direct determination of the neutral fat from the acetone soluble glycerol and its relation to the total and essential lipid content of tissues are demonstrated and discussed.

The essential lipid concentration of the various tissues is related to the extent and variety of their physiological activities and confirms a similar relationship previously demonstrated for the phospholipids, which comprise the largest fraction of the essential lipid in all the tissues studied. The distribution of the other lipid fractions, as well as the individual phospholipid components appears to be more directly related to the particular types of functions performed by the individual tissues.

An unexpected finding was the high concentration of cerebroside in cardiac and skeletal muscles, comprising in these particular muscles a much greater portion of the cellular lipids than cholesterol. Cerebroside was found to be absent from beef liver and turtle egg. There appears to be an indication of an inverse relationship between cerebroside and cholesterol, the concentration of these lipid components being reversed in the organ tissues, with the exception of brain, to that in skeletal and cardiac muscle. The distribution of sphingomyelin indicates that this particular phospholipid is of greater relative importance in the soft organs of the body than in skeletal muscles. The importance of the individual lipid constituents as well as the concentration of the essential cellular lipids in relation to the functional activity of various types of tissues is discussed.

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The Amino Acid Yield from Various Animal and Plant Proteins after Hydrolysis of the Fat Free Tissue

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In the past, the principle methods for estimating the nutritional value of proteins have been based on nitrogen balance, body weight, and nitrogen stored. However, since the classical work of Osborne and Mendel (Mendel, 1923), attempts to evaluate proteins on the basis of their amino acid composition have been made, but no considerable progress along this line could be expected until all the essential amino acids were known (Rose, 1938). It was, then, deemed of interest to investigate the quantities of the essential amino acids in a number of animal and plant proteins. In carrying out such analyses, it is generally recognized that some of the values presented may differ somewhat from values that may be obtained in the future by improved methods. However, the data make possible a comparison *inter se*, regardless of the intrinsic accuracy of the results, since uniform analytical methods were used.

The experiments described below should be considered primarily of an orienting nature to the problem of estimating the nutritional value of a protein.

Experimental

The procedures given below are those used in the preparation, purification, and analysis of the proteins, although variations in the methods were often required by the nature of a particular sample.

A. Animal Proteins

The tissues, after grinding, were extracted with acetone, hot alcohol, hot benzene, and ether, and dried in the air. The residue was then dried at 105°C. to constant weight and ground to a fine powder in the ball

mill when necessary. In the case of milk, the proteins were originally precipitated with trichloroacetic acid or by heat coagulation after acidification with acetic acid. The gelatin, meat scraps, tankage, menhaden meal, hoof meal, and hog hair were commercial samples.

B. Plant Proteins

Yeast: Four strains of yeast, grown under different conditions, were rendered starch-free, and the pressed cake was frozen with solid CO_2 and thawed several times until the cells were thoroughly broken up as seen under the microscope. The resulting syrup was dehydrated and the fat-free residue was dried at 105°C.

Wheat Gluten: Three samples of wheat flour and two of farina were each made into a heavy dough and the starch was washed out with cold water. The gluten preparations were dried at 100°C., ground, and extracted with hot benzene and with ether. The resulting light yellow powder was dried at 105°C.

Wheat Germ: A sample of commercial wheat germ meal was extracted with benzene and ether and dried at 105°C.

Corn: Two samples of yellow corn and one of white were ground and extracted with hot benzene and with ether. The defatted material was dried at 105°C. The starch was separated from the nitrogenous substances according to Csonka's (1937) method. The insoluble residues, which contained almost all of the nitrogen of the corn, were dried with cold acetone (a large volume should be used to prevent the extraction of zein), benzene, and ether, and finally at 105°C.

Corn Gluten: A sample of commercial corn gluten was extracted with benzene and with ether and dried at 105°C.

Corn Germ: The germ was freed from dried yellow corn by steeping the whole kernel in warm dilute H_2SO_3 and subsequent grinding while wet. The germ was removed by floatation, washed,[1] and dried. The whole germs were then extracted with acetone, benzene, and ether. After drying, the almost colorless material was ground in a coffee mill. Ninety grams of this material were added with mechanical stirring to a mixture of 4000 cc. of water, 500 cc. of ice, and 40 cc. of 10 N sodium hydroxide. The suspension was stirred mechanically at 0°C. for 5

[1] It is probable that certain soluble proteins are removed from the germ during steeping. However, as our interest was in the amino acid composition of corn germ meal as commercially available for feeding purposes rather than in the natural germ, no attempts were made to eliminate these losses.

hours. The insoluble material was centrifuged off and the cloudy supernatant liquid was filtered in the cold through folded paper. The protein was obtained from the clear filtrate by precipitation with acetic acid and heat coagulation. The precipitate was washed with hot water and dried with acetone and ether. Yield 14 g., equal to approximately 66 per cent of the nitrogen in the original germ.

Corn Albumins: These were prepared by precipitation from steep water with either ammonium sulfate, trichloroacetic acid, acetone, or tannic acid. The protein precipitates were further purified by extraction with acetone, benzene, and ether, and dried at 105°C.

Soy Bean: Commercial soy bean meal was extracted with acetone, hot alcohol, hot benzene, and ether, and dried at 105°C. before analysis.

Analytical Methods

Nitrogen was determined by micro or macro Kjeldahl methods. Sulfur was determined by the Parr bomb procedure. Cystine was estimated by a modification of the Folin phospho-18-tungstic acid method and by the Fleming-Vassel (1941) *p*-dimethyl-phenylenediamine procedure. Methionine was calculated from the non-cystine organic sulfur or estimated by the colorimetric procedure of McCarthy and Sullivan (1941). Arginine, histidine, and lysine were isolated as the monoflavianate, nitranilate, and picrate respectively, by a micro modification of Kossel's method (*cf.* Block and Bolling, 1940). The values given in the tables were corrected for small "overall losses" incident to the method. Tyrosine, tryptophan, and phenylalanine were estimated, after alkaline hydrolysis, by methods based on the procedures of Millon, Folin, or Lugg, and Kapeller-Adler respectively (*cf.* Block and Bolling, 1940). As phosphomolybdotungstic acid (Folin's reagent) is reduced by a number of substances besides tryptophan, care must be used in interpreting results obtained with this reagent. The more specific Millon-Lugg method gave similar values with the majority of the animal proteins, but only approximately one half the quantity of tryptophan, as shown by the Folin method, with some of the plant products. The Millon-Lugg values are reported in the tables. Threonine was estimated by oxidation to acetaldehyde with sodium periodate or lead tetraacetate (*cf.* Block and Bolling, 1940).

Valine, leucine, and isoleucine were estimated by a modification of the differential oxidation method of Fromageot (*cf.* Block and Bolling, 1940).

It should be recognized that the methods used for estimating the various amino acids are *not* of equal precision. Errors are introduced by the formation of inhibiting substances or the partial destruction or modification of certain amino acids during hydrolysis. Mechanical and solubility losses play a part also. The advantages and drawbacks of several of the above procedures have been discussed (Block and Bolling, 1943). However, it is our opinion that all are of value in a comparative investigation, and if used in conjunction with biological experiments, will permit the nutritional evaluation of food proteins and especially of their supplementary relationships; although they may not be entirely reliable as a means of estimating the absolute[2] amounts of amino acids present in the intact protein molecule.

In brief, the estimation of cystine may be subject to large losses during hydrolysis. There are unavoidable mechanical losses in the isolation of arginine, histidine, and lysine. Tyrosine can be estimated with a relatively high degree of accuracy, while tryptophan is subject to hydrolytic losses which may be compensated for by the production during hydrolysis of compounds which reduced the non-specific phosphomolybdotungstic acid reagent of Folin. The Lugg method gives values which may be equal or lower than those found by the Folin and spectrophotometric procedures (Ross, 1941; Devine, 1941). Phenylalanine and methionine estimations are subject to all the usual difficulties of a colorimetric method. Threonine is estimated by the yield of acetaldehyde formed by mild oxidation. The presence of other compounds which yield acetaldehyde or aldehydes which react with *p*-hydroxydiphenyl to yield a chromogen will interfere with the accuracy of these estimations. Valine and leucine estimations are probably subject to the greatest errors of any of the methods used (*cf.* Block and Bolling, 1943). The estimation of isoleucine, although still imperfect, can be carried out with greater accuracy than that of leucine and valine.

The analytical results are summarized in Tables I and II. Average values are given. Under the columns Valine, Leucine, and Isoleucine

[2] The accuracy of amino acid estimations can be considerably increased by the use of recovery experiments (Block and Bolling, 1940, 1943). This procedure is especially valuable in those instances where only relatively small quantities of protein are required for an individual analysis. In these cases, a sufficient number of estimations can be carried out with and without the added amino acid to permit statistical evaluation. This method is useful where highly purified proteins of constant composition are available and the data are to be used for considerations of structure.

TABLE I

Percentage Composition of Some Animal Tissue Proteins

Calculated to 16 per cent of Nitrogen

	Cow's milk	Gelatin	Fibrin	Hemoglobins	Serum Proteins	Whole Egg	Tankage[3]	Meat Scraps	Menhaden meal	Hoof	Hair	Muscle	Brain
Arginine	4.3	7.6	6.8[c]	3.5	5.8	7.0	5.5	7.0	5.9	10.4	8.9	7.1 (6.9)	6.6 (6.4)
Histidine	2.5	1.0	2.3[c]	7.6	2.6	2.4	2.7	2.0	2.4	1	1.0	2.2 (2.3)	2.6 (2.5)
Lysine	7.5	4.3	7.5[cd]	8.0	8.0	6.0	6.0	5.1	5.7	3.2	2.6	8.1 (8.1)	6.2 (6.0)
Tyrosine	5.3	0.2	5.1	3.0	5.4	5.0[a]	2.9	3.2	2.8	5	3.1	3.1 (4.3)	4.1 (5.1)
Tryptophan	1.6	0.0	3.7	1 to 2	1.7	1.6[a]	0.7	0.7	1.2	1.5	1.3	1.2 (1.4)	1.3 (1.6)
Phenylalanine	5.7	1.8[b]	5.9	6.7	5.4	5.6[a]	6.0	4.5	4.8	4	2.7	4.5 (4.9)	4.9 (5.8)
Cystine	1.2	0.1	1.9	0.5	3.6	2.1[a]	1	1.0	1	7.3	16	1.1 (1.1)	1.8 (1.7)
Methionine	2.8	0.8	2.6	1.4	2.1	3[a]	3	3	3			3.3 (3.1)	(2.8)
Threonine	4.6	1.5	7.9	6.8	6.3	4.9	3–4	4	5	5 to 6	6.4	5.2 (4.6)	5.8 (5.3)
Leucine	16.2 ±3.1	3.7 ±0.5	14.3 ±3.9	16.6 ±2.3	18	19.0 ±2.1	13		10	15 ± 2		12.1 ±1.1	13.4 ±2.2
Isoleucine	4.4 ±0.4	1.1 ±0.2	5.0 ±0.5	1.5 ±0.3	3	5.3 ±0.3	2–3		4	4 to 5		3.4 ±0.2	3.6 ±0.3
Valine	4.5 ±0.4	2.1 ±1.0	3.9 ±1.8	8.2 ±1.0	6	4.4 ±0.6	6		4	5 ±1		3.4 ±0.4	4.9 ±0.7
Sulfur	1.0	0.4		0.5	1.3	1.5	0.8	1.1	0.9			1.1 (1.1)	1.2 (1.1)

[a] Calculated from the average of the amino acid content of white and yolk proteins.

[b] Calculated from Gordon, Martin, and Synge (1943).

[c] Calculated from Bergmann and Niemann (1936).

[d] Calculated from Albanese (1940).

Values in parenthesis from Beach, Munks, and Robinson (1943).

[3] It should be remembered, however, that the amino acid composition of different samples of tankage of the same total protein content will vary with the price of gelatin. If gelatin is cheap, more collagen will be put into the tankage. This is but a single example of the well recognized inadequacy of evaluating feeds by total nitrogen estimations. It is obvious that their classification based on the quantities of amino acids available to the animal is the desired goal.

when whole numbers are given, an insufficient number of analyses were carried out for statistical evaluation, and the values are only roughly approximate. In those cases where the figures are given to one decimal place, the percentages were calculated statistically with twice the standard error. However, in common with other investigators in this field, we have often observed that highly reproducible results may be obtained by each of several methods, and yet the values so found may differ considerably among the different analytical procedures. Thus Ross (1941) found 2.0 per cent of tryptophan by the Millon-Lugg method and 4.5 per cent of this amino acid by the Shaw and McFarlane glyoxylic

TABLE II

Percentage Composition of Some Plant Proteins

Calculated to 16.0 per cent of Nitrogen

	Whole corn	Corn gluten	Corn germ	Corn albumins	Wheat gluten	Wheat germ	Yeast	Soybean
Arginine	4.0	3.1	6.8	5.4	3.9	6.0	4.3	5.8
Histidine	2.4	1.7	2.7	6.7	2.2	2.5	2.8	2.3
Lysine	2.5	1.1	5.8	low	1.9	5.5	6.4	5.4
Tyrosine	6.1	6.2	4.9	3.8	3.8	3.8	4.2	4.3
Tryptophan	0.7	0.6	1.3	0.7	0.8	1.0	1.4	1.5
Phenylalanine	4.5	6.6	5.6	1.7	5.5	4.2	4.1	5.4
Cystine	1.1	1.2	1.2	0.5	1.9	0.6	1.3	1
Methionine		5.5	2.6		3	2		2.0
Threonine	3.6	4.0	4.4	3.9	2.7	3.8	5.0	4.0
Leucine	21.5 ±2.4	24.7 ±3.7	16.3 ±3.1	11.3 ±4.1	12.0 ±2.6		13.2 ±2.6	6 to 8
Isoleucine	3.6 ±0.3	4.9 ±0.3	3.7 ±0.4	1.3 ±0.4	3.7 ±0.2		3.4 ±0.2	4
Valine	4.6 ±0.7	4.6 ±1.4	5.8 ±1.2	2.5 ±1.1	3.4 ±0.5		4.4 ±0.8	4 to 5
Sulfur	1.7	1.5	1.0	0.9	1.1		0.9	1.1

acid procedure in the same protein preparations. Similar discrepancies between the two methods have been found by Li, *et al.* (1941).

Results

The nutritive value of some of these animal and plant proteins were confirmed by feeding experiments. Thus it is seen that yeast, wheat germ, corn germ, and soy bean proteins, which we now know to be of good biological value in human and animal nutrition, yield a balanced, although not perfect, mixture of the essential amino acids similar to that found in some animal products. Corn, corn gluten, corn albumins, wheat gluten, keratins, and gelatin are deficient in one or more of the nutritionally indispensable amino acids. The latter are recognized as being proteins of poor quality when used alone, but they may have an important supplementary value.

The analytical figures given in Tables I and II confirm, in general, the results of Beach, *et al.* (1941, 1943), Csonka (1937, 1939), Padoa (1937), and others. In the analyses of plant proteins, it should be borne in mind that different varieties of the same plant may vary in their contents of the amino acids; Csonka (1937), Hamilton and Nakamura (1940), and Kik (1941).

We have had occasion to check the results of comparative amino acid analyses by feeding tests. It will be seen from Tables I and II that corn germ proteins and cow milk protein yield approximately the same *proportions* of the essential amino acids and, therefore, they should be

TABLE III

Nutritive Value of Corn Germ and Milk Proteins

Average	Diets		
	Whole milk powder N = 4.0 per cent	Corn germ meal (solvent extracted) N = 3.6 per cent	Corn germ meal (hot expulsion) N = 2.9 per cent
Weight gained	114 g. (80–148)	103 g. (74–143)	125 g. (88–171)
Food eaten	615 g. (416–764)	586 g. (478–652)	594 g. (324–808)
Nutritive value*	1.9	1.8	2.1

* Nutritive value is the gain in weight in 60 days divided by the protein consumed. All three rations were made up to contain 10 per cent of protein.

Values in parentheses indicate range.

Nine male rats were used on each diet. Each animal had a litter-mate on the other two diets.

interchangeable when fed on an equal nitrogen basis. This hypothesis was confirmed experimentally using feeding experiments on white rats with either corn germ or dried milk as the sole source of protein.

Table III summarizes the results of growth experiments carried out on nine groups of male rats which were all litter-mates. The different groups, however, were not of the same age when started on the experiment. All animals were kept on the regime for 60 days. The two samples of corn germ meal, solvent extracted and hot pressed, were obtained commercially and were not subject to any further treatment.

The diets were composed of 10 per cent protein (calculated from

6.25 × N), 5 per cent of crisco, 5 per cent of cod liver oil, 4 per cent of inorganic salts, and sucrose to make 100 per cent. Vitamins A, D, E, B complex, linoleic acid and choline were supplied separately three times a week. Other tests using maintenance and paired feeding techniques confirmed the finding that milk and corn germ proteins have approximately the same nutritive value for the rat. This is in complete agreement with the recent findings of Hove and Harrel (1943).

Discussion

In order to correct amino acid deficiencies in the diet, it is not necessary to add the required units in pure form. The same result can be

TABLE IV

Approximate Quantities of Essential Amino Acids in Grams Furnished per Kilogram of Meat, Milk, White Flour, Whole Wheat Flour, Corn Meal, and Soy Bean Flour

Amino acid	Fresh meat	Whole milk	White flour	Whole wheat flour	Whole corn meal	Soy bean flour
	g.	*g.*	*g.*	*g.*	*g.*	*g.*
Nitrogen	32	5.8	21	19	16	76
Arginine	14.0	1.6	5.1	3.9	4.0	25.2
Histidine	4.5	0.9	2.9	2.4	2.4	8.5
Lysine	16.2	2.7	2.5	3.2	2.5	22.8
Tyrosine	7	1.9	5.0	4.5	6.1	20.4
Tryptophan	2.6	0.6	1.1	1	0.7	7.1
Phenylalanine	9.5	2.1	7.2	6.8	4.5	25.7
Cystine	2.2	0.4	2.5	1.9	1.1	4.8
Methionine	6.4	1.0	4		5	9.5
Threonine	10.3	1.7	3.5	3.9	3.6	19.0
Leucine	24 ±2	6 ±1	16 ±2	15 ±4	22 ±3	35 ±5
Isoleucine	7 ±1	1.6 ±0.1	4.9 ±0.3	5.8 ±0.7	3.6 ±0.3	19 ±2
Valine	7 ±1	1.6 ±0.2	4.5 ±0.6	5.8 ±1.1	4.6 ±0.7	22 ±3

obtained more conveniently and economically by employing proteins or protein concentrates which contain the required amino acids. Amino acid analyses should greatly facilitate the preparation of balanced protein foods for human and animal consumption. Such studies may also bring to light plant proteins which can be used to replace a portion of the more expensive animal proteins of the diet. For example, the replacement in certain foods and feed mixtures of skimmed milk powder by the cheaper corn germ meal. Likewise special experimental diets deficient in or containing a superabundance of one or more of the dietary essential amino acids can be made up. Thus, corn albumins are comparatively rich in histidine, but are poor in lysine and phenylalanine;

while corn gluten is deficient in lysine, but is richly supplied with leucine, isoleucine, and valine. Wheat germ and corn germ proteins appear to be rather well balanced in their content of the essential amino acids and contrary to the prevailing impression that wheat proteins are superior to those of corn, it seems from these analyses that both should have approximately the same biological value (*cf.* Hove and Harrel, 1943).

The approximate quantities of nitrogen and the essential amino acids which would be furnished per kilogram of fresh meat, whole milk, patent flour, whole wheat, corn meal, and soy bean are presented in Table IV. The superiority of whole wheat flour, especially in lysine, over white flour and corn meal is evident. The value of corn to furnish relatively large quantities of methionine, leucine, isoleucine, and valine per unit of total protein should be noticed. Although corn meal contains approximately one fifth as much protein as soy bean flour, it yields half sa much methionine and leucine, but only one tenth as much lysine and tryptophan.

If the data on meat and flour proteins (Tables I, II and IV) are compared, it will be seen that flour proteins are only slightly deficient in tryptophan, threonine, and possibly valine, but they are markedly deficient in lysine. In fact, it appears from these analyses that the addition of lysine alone to wheat flour should double or triple its nutritive value.

Conclusion

Milk, gelatin, fibrin, hemoglobin, serum, egg, tankage, meat scraps, fish meal, hoof meal, hair, muscle, brain, corn, corn gluten, corn germ, corn albumins, wheat gluten, wheat germ, yeast, and soy bean have been analyzed for nitrogen, sulfur, and eleven amino acids. Except for gelatin, hemoglobin, and keratin, the animal proteins, when calculated to 16 per cent of nitrogen, yield approximately 1 per cent of sulfur, 1–2 per cent of cystine, 5–7 per cent of arginine, 2–3 per cent of histidine, 5–8 per cent of lysine, 3–5 per cent of tyrosine, 1–2 per cent of tryptophan, 5–6 per cent of phenylalanine, 4–6 per cent of threonine, 4–6 per cent of valine, 10–20 per cent of leucine, and 3–5 per cent of isoleucine. Yeast, corn germ, wheat germ, and soy bean yield approximately the same proportions of these amino acids. In contrast, gelatin, hemoglobin, keratins, corn, corn gluten, corn albumins, and wheat gluten, are poorly balanced with respect to one or more of the essential amino acids.

The use of amino acid analyses for the nutritional evaluation of proteins, for the compounding of foods and feeds, and for other purposes, is mentioned.

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The Effect of Different Sodium Chloride Concentrations on Nuclei from Chicken Erythrocytes*

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Introduction

Mirsky and Pollister (1) found that nuclei of liver cells entirely lost their staining capacity after the slice of liver had been treated with molar NaCl. Dounce (2) found that isolated liver nuclei treated with 5 per cent NaCl were only partially soluble, and that the insoluble residue could be centrifuged with a high speed centrifuge (15,000 R.P.M.). Laskowski (3) observed that the nuclei suspension obtained from chicken erythrocytes by the lysolecithin method produced a solid gel when treated with a strong solution of NaCl. When treated with water an agglutination of the nuclei occurred. Later it was observed microscopically that the cellular framework did not disappear in either case. When stained with aqueous methylene blue the debris of nuclei could be seen to be included within a net of the nucleoprotein threads. Essentially the same picture could be seen after staining with the Feulgen technique. The picture was characteristic, resembling an "artificial connective tissue." It was decided, therefore, to investigate systematically the microscopical appearance of nuclei in NaCl solutions of different concentrations.

Experimental

Nuclei from chicken erythrocytes were prepared according to the previously described method (3). Both bee stingers and snake venom were used as sources of lecithinase A.[1] The Dounce and Lan (4) modifi-

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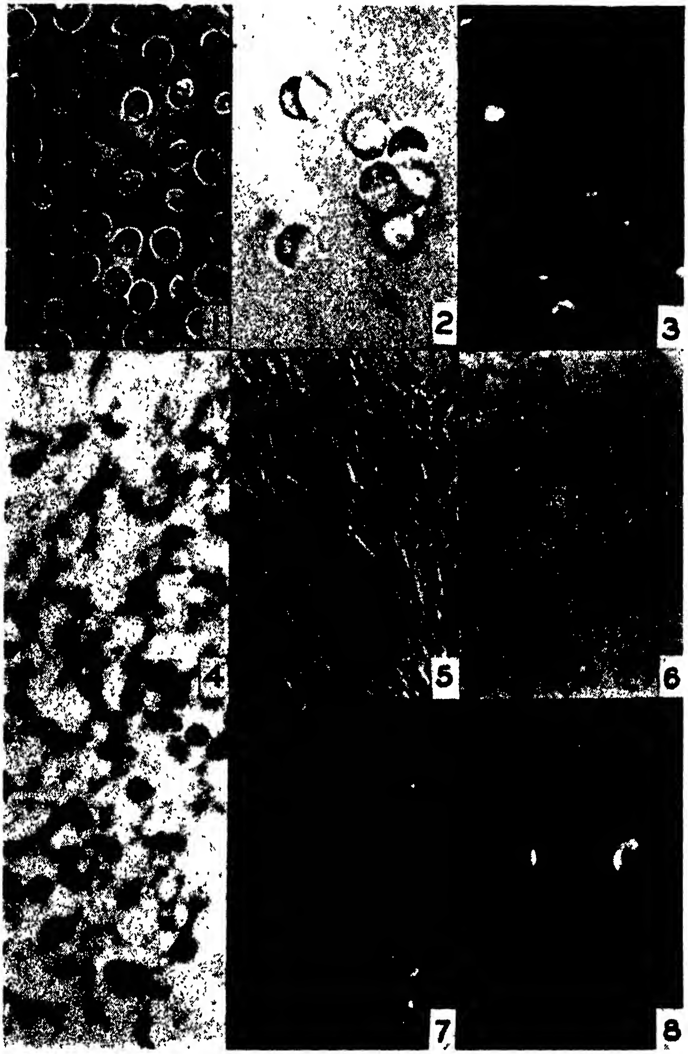
[1] The sample of purified lecithin (cadmium salt) was kindly supplied by Dr. A. Scharf, American Lecithin Co., Inc.



Figs. 1–8

PLATE

Photomicrographs of chicken erythrocytes and preparations of nuclei from hemolyzed cells.

Hemolysis with lysolecithin, except Fig. 2; all wet mounts except Fig. 4. Oil immersion objective, 5 × ocular.

See bottom of facing page for legends

cation was also used and found to be advantageous in shortening the time of hemolysis and in facilitating the washing. A few other hemolytic agents were tried, including the "wetting agents"; the results were not satisfactory due to the agglutination of nuclei occurring simultaneously with hemolysis.

Dounce and Lan (4) noted that nuclei obtained either with lysolecithin or with saponin possessed stromata which could be shown after heavy staining (crystal violet). This finding was confirmed (Fig. 1). However, the number of nuclei possessing apparently complete stromata was different in different preparations, as also was the degree of damage to the stromata. We were unable to correlate these differences with any difference in technique of preparation. We can not, however, agree with Dounce and Lan that nuclei obtained with saponin have a better microscopic appearance than those obtained with lysolecithin. When the nuclei in our preparations were examined immediately after lysing with saponin they appeared normal; but after subsequent centrifugation we always found several crescent-shaped nuclei, located at the periphery of the stroma (Fig. 2). No crescentic nuclei were found in lysolecithin preparations.

We did not find supporting evidence for the suggestion of Dounce and Lan that agglutination of nuclei may be due to the complete removal of stromata. Stable preparations were obtained in which only about 20% of the nuclei showed stromata. In the experiments described below agglutination was noted in 0.6 per cent and 3 per cent NaCl solutions when stromata were still present.

Fig. 1. Unstained nuclei in 1 per cent NaCl; 'normal' nuclei and stromata.

Fig. 2. Unstained nuclei in 1 per cent NaCl; crescent-shaped nuclei after centrifugation of a saponin preparation.

Fig. 3. Unstained nuclei in 10 per cent NaCl; darkfield; uniformity of the dispersed phase of dissolved nucleoprotein between the still present nuclear outlines.

Fig. 4. Nuclei in 10 per cent NaCl, Helly fixation, stained by the Feulgen method to show precipitation of the nucleoprotein.

Fig. 5. Nuclei in 0.1 per cent NaCl, stained with crystal violet to demonstrate precipitation of nucleoprotein as an "artificial connective tissue."

Fig. 6. Normal erythrocytes in 5 per cent NaCl, stained with crystal violet to show presence of stainable particles outside the nucleus.

Fig. 7. Unstained nuclei in 4 per cent NaCl; darkfield; nuclear and stromal 'membranes' still present.

Fig. 8. Unstained nuclei in 0.2 per cent NaCl; darkfield; nuclear and stromal 'membranes' still present.

Of the several possible factors affecting the extraction of nucleoproteins from nuclei, only one, the effect of salt (NaCl) concentration, was investigated. It had been previously noted that pH markedly influenced the stability of the nuclei suspension. Agglutination and gelatination occurred even in isotonic solutions at pH values above 8.5. Briggs and Laskowski (5) found that in the region of pH from 2 to 5, the suspensions of nuclei in 0.1 *M* buffers were sufficiently stable to allow a determination of the mobility curve. The electrophoretic cell of Briggs (6) was used. The isoelectric point of nuclei prepared with lysolecithin was found to lie between pH 3.1 and 3.2.

Small quantities of the stock nuclei suspension in 1 per cent NaCl were added to NaCl solutions to make percentage concentrations of the salt of 10, 5, 4, 3, 2; 0.8, 0.6, 0.4, 0.2, and 0.05. The resultant suspensions contained relatively few nuclei for best examination of the individual components in the microscopic field. In such high dilutions of nuclei a solid gel was not formed, even in the strong salt solutions. The phenomenon observed could be called rather an agglutination; the remains of nuclei were included in the gelatinized mass. With vigorous shaking the mass could be broken and a viscous sol obtained. In the high dilutions of nuclei the macroscopic appearance of clumps of agglutinated nuclei was almost identical at both ends of the series of salt solutions. Agglutination occurred readily in 3% and stronger, and in 0.6% and weaker, solutions.

The typical microscopic picture in these intermediate salt concentrations (2 and 3%, 0.8 and 0.6%) showed the extension of nuclear material within the stromal outline; at the same time the nuclear outlines were less sharp. This observation was interpreted as representing a gradual hydration of nucleoprotein complex, leading finally to formation of a sol. The limitation of this extrusion to the stromal limits may be due either to the adsorption of nucleoprotein on the surface of the stroma protein, or else due to the mechanical resistance offered by the stroma to the increasing volume of the hydrated nucleoprotein.

In the higher and lower NaCl concentrations the internal nuclear structure disappeared, and considerable amounts of nucleoprotein were present as sol outside nuclear and stromal outlines. The sol was uniform in dark-field examination (Fig. 3). When the preparation was stained (either with crystal violet, methylene blue, or by the Feulgen method) the nucleoprotein was partially precipitated and appeared as a net of fibers enclosing the nuclear debris (Figs. 4 and 5). In unstained prep-

arations nuclear and cellular outlines were clearly shown in dark-field illumination (Figs. 7 and 8).

No solution investigated produced a visible rupture of a nuclear membrane; nevertheless, nucleoprotein was extracted from within the nucleus, both in high and low NaCl concentrations. This indicates that, in these cells, a selectively permeable nuclear membrane does not exist.

The appearance of erythrocyte nuclei in 5 or 10 per cent NaCl solutions differed slightly from the description of Mirsky and Pollister (1) for liver cell nuclei. In our case part of the "supporting structure" persisted at these concentrations, and it still contained some nucleoprotein as shown by a positive Feulgen reaction. The observed difference may be due to lack of such a structure in liver cells, or the cytoplasm present may have masked such a structure.

As a control experiment, intact erythrocytes were placed in 3 per cent and stronger salt solutions (Fig. 6). Colored granules were present in the cytoplasm after staining with crystal violet. Thus, in accord with Mirsky and Pollister, the passage of nucleoprotein from nucleus to cytoplasm was observed even in intact cells. It seems possible that such passage may be responsible for the small amounts of desoxy-ribonucleic acids reported to be present in the cytoplasm. Since hemolysis occurs in the diminished salt concentrations, a similar comparison can not be made to erythrocytes in such media.

At a pH value near 7, the structure of the erythrocyte nucleus can be preserved only within rather narrow limits of salt concentrations (near 1%). This can not be generalized to apply to all nuclei. Dounce (2) obtained liver nuclei by grinding the tissue with 5 volumes of water at pH 6; the salt concentration in his case was probably below 0.2 per cent NaCl. Even at pH 6 the erythrocyte nuclei will not stand such a low salt concentration without marked changes. Although we realize that nuclei from various sources have different characteristics, these findings may be of some general applicability. One may assume that whenever a tissue extract is made with physiological saline only insignificant quantities of nuclear constituents will dissolve. On the other hand, when tissues are extracted with higher or lower NaCl concentrations, an increase in the content of desoxy-ribose nucleoprotein in the extract may be expected. This assumption has been confirmed by one of us in the case of saline extracts of pig kidney.

From the microscopic examination of nuclei in the salt solutions of different strengths, the conclusion was drawn that the process of dis-

integration of nuclear structure was gradual. An attempt was made to secure fractional extracts of the nucleoproteins, but it was not successful. In 2 and 3 per cent NaCl solutions the nuclei could be centrifuged down easily, but the supernatant contained only traces of nucleoprotein. In the higher concentrations, a gel was formed so that centrifugation with the ordinary centrifuge failed to separate the components.

It still was possible, without a high speed centrifuge, to obtain the nucleoprotein solution almost free from the morphological constituents by extracting the nuclei with 10 volumes of 5 per cent NaCl solution stirred in the Waring blender. The gel so obtained could be filtered through a Büchner funnel covered with a thick layer of celite 545. The viscous nucleoprotein solution thus obtained accounted for two-thirds to three-fourths of the total nuclear material on the basis of dry weight. The soluble nucleoprotein exhibited the same properties as the "plasmosin" of Bensley (7) and the nucleoprotein described by Mirsky and Pollister (1). It could be divided into two fractions: water soluble and water insoluble. Both fractions were insoluble in 0.9 per cent NaCl, giving a characteristic thread-like precipitate, which changed into a gelatinous mass after standing.

The observation of Mirsky and Pollister that the nucleoprotein which has been once dissolved in water loses partially the thread-like appearance was also confirmed. Furthermore, it was noticed that the damaging effect of water was greater when applied directly to the nuclei than when applied to the 5 per cent NaCl extract. In the first case, by raising the concentration of NaCl to 0.9%, no threads whatsoever were produced. In the second case, after dialysis until salt-free, the water soluble nucleoprotein still exhibited a considerable degree of the thread-like structure when precipitated with 0.9 per cent NaCl.

Summary

1. Chicken erythrocyte nuclei treated with NaCl concentrations from 1 to 10% showed a gradual solution of nuclear content. Under our experimental conditions it was not possible to dissolve the nuclei completely. The nuclear framework could still be demonstrated.

2. Nuclei treated with NaCl concentrations from 1 to 0.05% also showed loss of the nucleoprotein due to solution. In these salt concentrations also the nuclear framework remained.

3. The phenomenon observed in both increasing and decreasing salt concentrations was essentially the same, namely, gradual solution of

nucleoprotein. Since the nucleoprotein was extracted from nuclei which showed no visible rupture of the nuclear "membrane," it may be that a selectively permeable nuclear membrane did not exist in these cells.

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Induced Polycythemia in Salamander by Cobalt, Ascorbic Acid, and Other Water Soluble Vitamins[1]

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Introduction

In salamander, the hemopoietic function is sharply separated, erythrocytes and thrombocytes being differentiated in spleen and granulocytes in the liver. Even after total splenectomy the lymphogranulocytopoietic center does not become erythropoietic but the function of the red cell formation is taken up by the heart and the general circulation, Jordan and Speidel (1). Such a division of the hemopoietic function is interesting and affords a ready observation on the effect of the substances tested on red cell formation. So far as the authors are aware, the influence of vitamins on the formation of red cells in salamander was first studied by Slonimski (2) in 1938. Slonimski found that the administration of vitamin C in excess into the embryo of *Ambystoma mexicana* does not accelerate the first appearance of hemoglobin; however it influences the differentiation of the red blood cells and increases their number in later stages.

Polycythemia induced by cobalt has been produced in various laboratory animals here, namely, mice, rabbits, fowls, rats, dogs, guinea pigs, and frogs, probably not yet in salamanders.

The present attempt is to induce polycythemia in salamander, *Triturus orientalis Davis*, by intraperitoneal injection of cobaltous chloride, ascorbic acid, and some other water soluble vitamins, the administration of which has brought about some sort of hemopoietic response in other laboratory animals.

[1] The crystalline vitamins used in this study are gifts to one of the authors from his friends at Cornell University.

Experimental

Experiment I

The animals used in this experiment were collected from the Kunming Lake and kept in a big wooden container of water with water plants. No additional food was supplied. The red cell count of the animals gradually decreased during captivity probably due to partial starvation or changes of reproductive state. During the experiment, animals of the same group were put in one large beaker with water but without food. Twenty-nine animals which varied within narrow ranges in body size were divided into seven groups, one untreated group and six treated groups: Each treated group was given one of the six kinds of water solutions, *i.e.* thiamin, riboflavin, pyridoxin, nicotinic acid,

TABLE I

Red Cell Counts and Hemoglobin Value of Salamander

Solution injected	Number of animals used	Red cell count (*thousand cells/cmm.*)	Hemoglobin (%)
Thiamin	2	197 (196–198)	85
Riboflavin	4	162 (124–186)	80
Pyridoxin	4	145 (110–160)	76
Nicotinic acid	4	173 (124–200)	83
Ascorbic acid	4	173 (130–216)	77
$CoCl_2$	4	168 (130–200)	84
Untreated	7	151 (110–176)	69

ascorbic acid, and cobaltous chloride. All the vitamin solutions contained 10 milligrams per 100 cc. water. For cobaltous chloride a 0.005 per cent solution was used. Injection was accomplished with a 2 cc. syringe. The animal was turned abdomen up and an injection of 0.2 cc. of the solution was given through the abdominal wall into the coelom. The treatment was continued daily for 36 days. On the 37th day the animals were killed, and blood was taken directly from the heart for red cell count and hemoglobin determination. For red cell count, the blood sample was diluted in a pipet 1:200 with Hayem's solution and two counting chambers were used. For hemoglobin determination, the colorimetric acid-hematin method was adopted, and the relative hemoglobin value was expressed in percentage as read from hemometer for human blood.

In Table I, the results of the experiment were summarized.

Experiment II

As cobalt and ascorbic acid are the two exerting definite influence on red cell formation in other animals as reported by many investigators and they are closely related in cobalt polycythemia as proposed by Barron and Barron (3) and supported by Davis (4), they are chosen for further study.

Forty-eight animals with body weight from 6 to 13 grams were used. They were divided into three groups with the sexes separated. The cobalt group was given daily an intraperitoneal injection of 0.2 cc. of 0.005 per cent solution of cobaltous chloride. As the duration of the experiment was thirty-one days, the total amount of cobaltous chloride each animal received was 0.31 milligrams. The ascorbic acid group was given an intraperitoneal injection of 0.2 cc. of 200 mg. per cent ascorbic acid solution, the strength of which was constantly checked by indophenol titration. By the end of the experiment, each animal in this group received about 9.55 mg. of ascorbic acid. The control group, instead of being untreated as in the first experiment, was given daily an intraperitoneal injection of 0.2 cc. distilled water. The red cell count and hemoglobin value obtained from the animals of the three groups are tabulated in Table II.

The differences between the mean red cell count of treated and control female animals are 39 thousand cells per cmm. blood for ascorbic acid group and 29 thousand cells per cmm. blood for cobalt group. As tested by Fisher's "t" method (5) the "P" value is 0.01 (in case of cobalt group) or less (in case of ascorbic acid group) which means that only one-hundredth or less is the chance that these differences occur as errors of observation. Similarly, the differences between the relative hemoglobin value of the female treated and the female control groups are also significant with a "P" value less than 0.01. On account of the smaller number in the male control group, the results are less obvious. Therefore, it can be stated that both ascorbic acid and cobaltous chloride increase the red cell count and the relative hemoglobin value in female salamanders and probably in males too.

Histological Examination

Blood smears and spleen smears were made out of the fresh materials and were treated with Wright's stain. The spleen was fixed with Helly's solution and stained with Delafield hematoxylin and azur-eosinate.

Microscopic examination of the blood smears of the three groups shows that younger stages of erythrocytes are rare and the mitosis, seen rarely in leucocytes, is not seen at all in red cells. General blood circulation thus plays little part in erythropoiesis in these animals.

Sections of spleen of all the three groups show no pathological picture.

TABLE II

Red Cell Count and Hemoglobin Value of Salamanders Treated with Cobaltous Chloride and Ascorbic Acid

(R.B.C. in thousands/cmm. blood; hemoglobin in %)

Male no.	Control group *R.B.C.*	*Hb.*	$CoCl_2$ group *R.B.C.*	*Hb*	Ascorbic acid group *R.B.C.*	*Hb*
1	128	60	150	70	126	
2	101	60	142	62	194	78
3	152	72	158	94	155	65
4			236		157	65
5			178		154	64
6			130	60	175	71
7			135	66	162	83
Average ...	127 ±9.9	64 ±2.7	161 ±9.3	70 ±4.1	160 ±5.3	71 ±2.2
Female no.	**Control group *R.B.C.***	***Hb***	**$CoCl_2$ group *R.B.C.***	***Hb***	**Ascorbic acid group *R.B.C.***	***Hb***
1	116	55	126	50	184	89
2	125	60	172	70	130	65
3	113	56	149	72	190	70
4	150	55	142	60	175	70
5	125		175	64	174	75
6	144	66	187	67	133	65
7	109	50	141	78	166	80
8	95	50	172	70	149	70
9	122	58	134	63	146	65
10	131	60			198	71
11	103	58				
12	183					
Average ...	126 ±4.6	57 ±1.0	155 ±4.8	66 ±1.8	165 ±5.1	72 ±1.6

$$P.E._m = \pm 0.6745\sqrt{\frac{\Sigma D^2}{N(N-1)}}.$$

More red blood cells and less lymphocytes are found in cobalt and ascorbic acid treated animals. In these treated groups the whole section of the spleen is packed chiefly with young erythrocytes and the larger part of the lymphocyte contents is gone. This is supposed to be a sign of a real, more active cell formation rather than the result of any ab-

normal spleen dilatation, because the conditions of the small blood vessels in the spleen sections of all the three groups are the same, *i.e.* they are not particularly constricted in control group or dilated in treated groups. Though mitosis is not found in all the three groups, amitosis is commonly seen and appears to be more or less abundant in cobalt- or ascorbic acid-treated animals.

Discussion

The result in the present study has shown a distinct increase in the red cell count in animals treated with cobalt or ascorbic acid. Since histological examination of the spleen, the erythropoietic center of salamander, shows that an increased rate of red cell formation does exist, this increased rate of the formation of red cells in spleen may be regarded as a cause of the rise in red cell count and in hemoglobin value of the animals treated with either cobalt or ascorbic acid.

The spleen sections and smears in the treated as well as the control groups show that the forerunners of red blood cells are multiplied by amitosis and not by mitosis. Whether amitosis in red cell series occurs normally in well-fed animals or only in starved or partially starved animals as used in the present study is a question for further investigation.

Summary

Polycythemia has been induced in salamander, *Triturus orientalis Davis*, by intraperitoneal injection of ascorbic acid and dilute cobaltous chloride solutions. Histological examination shows that spleen, the main organ for erythropoiesis is activated. The other water soluble vitamins, may induce polycythemia in the salamander.

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Microbiological Aspects of Streptothricin
II. Antibiotic Activity of Streptothricin

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Introduction

The discovery of streptothricin formation by *Actinomyces lavendulae*, the preparation of crude concentrates of the active material, and some of its antibacterial properties have recently been published (1, 2). A note on an assay method developed out of the present work has already appeared (3). The above-named papers may be consulted for additional details. Certain irregularities in the behavior of some streptothricin preparations observed in the course of assays for their relative content of the active agent indicated the need for a systematic study of the antibiotic activity of streptothricin.

Experimental

Some antibiotic agents of microbial origin, particularly tyrothricin and penicillin, are inhibitory principally for Gram-positive bacteria. Streptothricin is active against a variety of bacteria irrespective of their Gram-negative reaction (1). It is not, however, alone in its effectiveness against Gram-negative bacteria; other agents, including claviformin (4), clavacin (5), pyocyanase (6), actinomycin (7), penicillic acid (8), fumigatin (9), and others (10), act similarly. *Escherichia coli*, long considered highly resistant to the action of antibiotic substances, is susceptible to streptothricin. Certain closely related Gram-positive aerobic spore-formers, such as *Bacillus subtilis*, *B. mycoides*, *B. cereus*, and *B. megatherium*, range from high sensitivity to nearly complete resistance to streptothricin.

Antimicrobial Activity of Streptothricin and Penicillin

Bacteria. A more complete study of the antimicrobial properties of streptothricin has now been made and extended to include, besides the

bacteria, some yeasts, and pathogenic and saprophytic fungi. Of special interest is the comparison with penicillin activity against several bacteria which is presented graphically in Fig. 1 to bring out more clearly the relative differences in the susceptibility of the different bacteria to these two agents. A comparison of this type may be called a bacterial inhibition spectrum. The sensitivity of the Oxford strain of *Staphylococcus aureus* (H) is taken as a base against which the sensitivities of the other bacteria are compared. Inhibitory concentrations were determined by

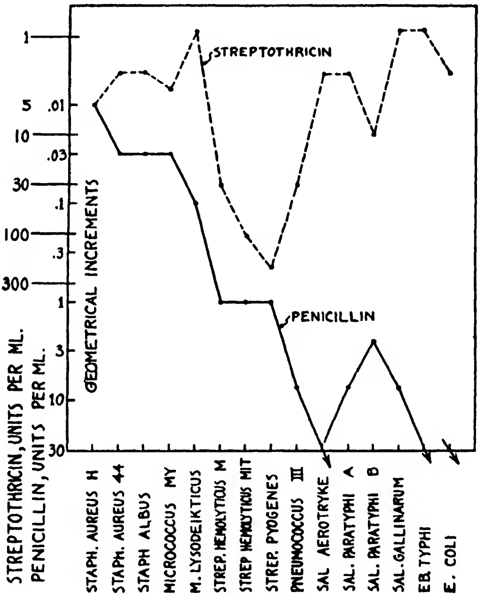

FIG. 1
Bacterial Inhibition Spectrum of Streptothricin and Penicillin

streaking the different test bacteria on nutrient agar containing various levels of the antibiotic substances. For reasons discussed elsewhere, the penicillin unit (11, 12) and the streptothricin unit (1, 3) are used to express the potencies of these substances. These units have no relation to each other, and, therefore, the absolute values are not comparable. However, the sensitivity of each organism relative to *S. aureus* (H) affords a basis for evaluating the respective efficacy of streptothricin and penicillin. Fig. 1 shows that penicillin is effective mainly against staphylococci and significantly less so against streptococci. The Gram-negative bacteria are markedly resistant since over 3,000 times the

amount of penicillin required to inhibit the staphylococci had no effect upon *E. coli*, *Eberthella typhi* and *Salmonella aerotryke*.

The two curves in Fig. 1 show that the action of streptothricin is not so limited against these bacteria and is quite different from that of penicillin. Interestingly, the streptococci as a group appear to be more resistant to streptothricin than the staphylococci. The susceptibility of the Gram-negative bacteria is in sharp contrast to penicillin.

Yeasts. Table I summarizes the inhibiting action of streptothricin upon the growth of 12 species of yeasts of different genera. Sensitivity was determined by streaks on yeast extract—1 per cent dextrose agar. A variety of different yeasts are susceptible to streptothricin, some being of the same order of sensitivity as *E. coli* used as a control. *Dipodascus uninucleatus* is appreciably more sensitive to streptothricin than *E. coli*, whereas the other yeasts showed different degrees of resistance. Three of the 12 species tested were not inhibited at 160 units per ml., the highest concentration used, which was 32 times that required for inhibition of *E. coli*. Penicillin, on the other hand, was entirely inactive against all the yeasts tested in levels up to 30 Oxford units per ml., a level 3000 times that required to inhibit *S. aureus* (H). Higher levels were not tested.

Pathogenic fungi. The pathogenic fungi, which are yeast-like in nature, have a spectrum somewhat similar to the yeasts, but as a group are more resistant to streptothricin than the yeasts. Certain forms, such as *Blastomyces dermatiditis* and *Achorium schoenleinii*, are inhibited in ranges which compare favorably with *E. coli*. Others are unaffected at about 100 times that required to inhibit *E. coli*. All of the pathogenic fungi tested were unaffected by 30 Oxford units/ml. of penicillin.

Saprophytic fungi. Representatives of a number of genera of saprophytic fungi showed, for the most part, considerable resistance to streptothricin although some were definitely inhibited, one being just as sensitive as *E. coli*. Seven of nineteen were inhibited at levels below 300 units per ml. after 48 hours incubation on peptone-glucose agar. After an additional 48 hours incubation, readings were higher in most cases, indicating the action of streptothricin is delaying and fungistatic, rather than fungicidal. Penicillin had no effect upon any of the fungi in quantities 10,000 times that necessary to inhibit *S. aureus* (H).

These groups of representative microorganisms provide evidence for the rather general inhibitory action of streptothricin. In other words, streptothricin is antimicrobial rather than merely antibacterial. Certain other agents, namely, actinomycin (7) and gliotoxin (13), may also be classified in the antimicrobial group.

TABLE I

Inhibition Spectrum of Streptothricin on Yeasts and Fungi

Organism	Streptothricin units per ml.									
Yeasts[1]	0	0.64	1.25	2.5	5	10	20	40	80	160
Dipodascus uninucleatus	+[2]	+	0	0	0	0	0	0	0	0
Sporobolomyces roseus	+	+	–	0	0	0	0	0	0	0
Debaryomyces guilliermondii	+	+	+	+	–	0	0	0	0	0
Torulaspora delbrückii	+	+	+	+	+	0	0	0	0	0
Schwanniomyces occidentalis	+	+	+	+	+	0	0	0	0	0
Torulopsis menosa	+	+	+	+	+	–	0	0	0	0
Rhodotorula rubra	+	+	+	+	+	–	0	0	0	0
Saccharomyces cerevisiae	+	+	+	+	+	+	–	–	0	0
Zygosaccharomyces marxianus	+	+	+	+	+	+	–	–	0	0
Zygopichia californica	+	+	+	+	+	+	+	+	+	+
Mycoderma valida	+	+	+	+	+	+	+	+	+	+
Candida stellatoidea	+	+	+	+	+	+	+	+	+	+
E. coli (control)	+	+	+	+	0	0	0	0	0	0
Pathogenic Fungi[3]	0	.03	.1	.3	1	3	10	30	100	300
Blastomyces dermatiditis	+	+	+	+	+	+	0	0	0	0
Achorium schoenleinii	+	+	+	+	+	+	+	–	0	0
Epidermophyton inquinale	+	+	+	+	+	+	+	+	–	–
Trichophyton interdigitale	+	+	+	+	+	+	+	+	+	0
Candida albicans	+	+	+	+	+	+	+	+	+	+
Microsporum felineum	+	+	+	+	+	+	+	+	+	+
Trichophyton gypseum	+	+	+	+	+	+	+	+	+	+
E. coli (control)	+	+	+	+	+	–	0	0	0	0
Saprophytic Fungi[4]	0	.03	.1	.3	1	3	10	30	100	300
Rhizopus nigricans	+	+	+	+	+	+	0	0	0	0
Penicillium notatum	+	+	+	+	+	+	+	0	0	0
Paecilomyces sp.	+	+	+	+	+	+	+	0	0	0
Fusarium sp.	+	+	+	+	+	+	+	0	0	0
Chaetomium sp.	+	+	+	+	+	+	+	+	0	0
Spondylocladium hylogenum	+	+	+	+	+	+	+	+	0	0
Metarrhizium sp.	+	+	+	+	+	+	+	–	–	0
Acrothecium robustrum	+	+	+	+	+	+	+	–	–	–
Sepedonium sp.	+	+	+	+	+	+	+	+	–	–
Dematium sp.	+	+	+	+	+	+	+	+	–	–
Papulospora sp.	+	+	+	+	+	+	+	+	+	+
Syncephalastrum sp.	+	+	+	+	+	+	+	+	+	+
Sterigmatocystis sp.	+	+	+	+	+	+	+	+	+	+
Aspergillus flavus	+	+	+	+	+	+	+	+	+	+
Trichoderma sp.	+	+	+	+	+	+	+	+	+	+
Circinella spinosa	+	+	+	+	+	+	+	+	+	+
Mucor sulfa	+	+	+	+	+	+	+	+	+	+
Gliocladium deliquescens	+	+	+	+	+	+	+	+	+	+
Neurospora ascosporia	+	+	+	+	+	+	+	+	+	+
E. coli (control)	+	+	+	+	+	–	0	0	0	0

[1] YED agar; 4 days' incubation, 30°C. We wish to thank Dr. Emil Mrak for these cultures.

[2] + = good growth, – = partial growth, 0 = no growth.

[3] YED agar; 4 days' incubation, 30°C.

[4] Peptone-glucose agar; 2 days' incubation, 30°C. We wish to thank Dr. C. Thom for supplying most of these fungi.

Combined Action of Streptothricin and Penicillin

The wide differences in bacterial specificity of streptothricin and penicillin raises the interesting, if not practical, question as to their combined effect on those bacteria which happen to be sensitive to each of the agents singly. Data from rough preliminary experiments suggested that levels which were subinhibitory when acting alone become inhibitory when acting in concert. The effect did not appear to be more than additive. From the fact that subinhibitory levels become inhibitory when acting together, it might be adduced that both these agents have an affinity for a common mechanism in the cells or, as an alternative, for two different secondary mechanisms, which in turn are linked to the same primary mechanism. The fact that the two agents are so selective in their action against different bacterial species speaks against the first possibility. A more quantitative analysis of this effect in liquid media (nutrient broth) employing the turbidimetric method (14) yielded results presented in Fig. 2, Part A. The control curve for penicillin alone is the typical logarithmic inhibition previously described for carbohydrate-free media (14, 15). The inhibition by streptothricin is, however, quite different. A slight gradual inhibition occurs over the range of low concentrations of streptothricin and is followed by a sudden sharp break indicating a much greater rate of inhibition. This increased rate is linear until complete inhibition. There appears to be little effect up until a critical concentration (in this experiment 1.5 units per ml.), after which there is marked inhibition which is directly proportional to the streptothricin concentration. The negligible inhibition provided by the lower concentrations is interpreted below.

The combined action of penicillin and streptothricin is almost quantitatively the sum of inhibitions of both acting singly as is shown in Fig. 2, Part B. The experimentally obtained curve is virtually identical with that calculated by addition of the inhibitions obtained singly (Fig. 2, Part A). The inhibition distances for the streptothricin in this experiment were relatively small compared to the total, but the 1.0 unit level was quite satisfactory for calculation purposes. For example, the inhibitions caused by streptothricin and penicillin acting singly and represented by the Distance X and Y in Part A is equal to Distance Z in Part B when those same amounts of streptothricin and penicillin acted simultaneously. The addition theory was checked further by the total inhibition obtained with random mixed concentrations of the two agents, results of which are given in Part C of Fig. 2. The calculated curve follows fairly well that obtained experimentally although the trend is

somewhat less than quantitatively additive. Distances R and S obtained singly (Part A) gave Distance T (Part C). Experimentally, the combined actions fell short of being quantitatively additive; they were, however, decidedly greater than either alone. The discrepancy between the calculated and observed values may be due to the presence of some cells with different degrees of resistance to the two different antibacterial

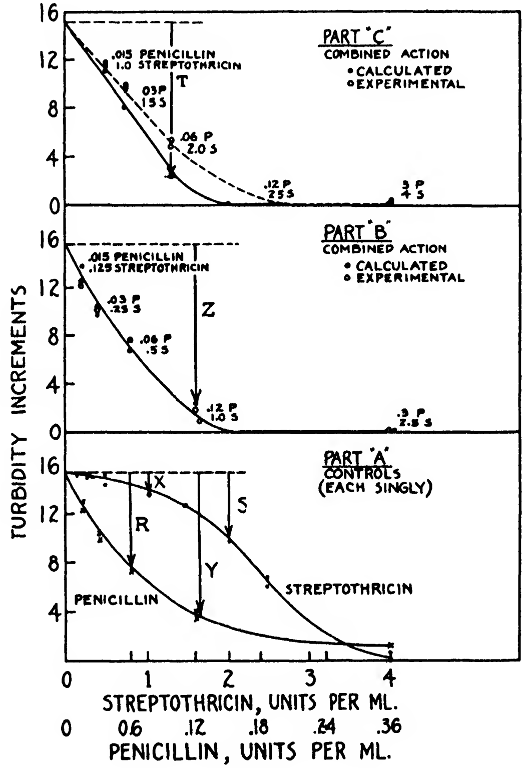

Fig. 2

Combined Inhibitory Action of Streptothricin and Penicillin

agents. These results are not contrary to the idea of common inhibition expressed above.

Acquired Resistance to Streptothricin

Numerous instances of acquired resistance of various bacteria to certain antibacterial agents (11, 16) make it probable that they would

similarly acquire resistance to streptothricin. Actually, the resistance of *E. coli* to streptothricin increases as a result of daily serial transfer in streptothricin-containing media. After 6 transfers, *E. coli* grows in a medium containing 70 units per ml., whereas a control unadapted culture was inhibited by 1 unit per ml.

The augmented resistance of *E. coli* may be due to selection of resistant cells present in the original culture. Resistant cells in this strain persist over an unexpectedly large range of concentrations of the inhibiting agent; at times several hundred per cent more streptothricin is required for complete inhibition than is required for partial inhibition. Single colonies were selected by plating and 24 hour nutrient broth cultures of these isolates streaked in the usual manner on streptothricin nutrient agar plates. There was considerable variation in numbers of

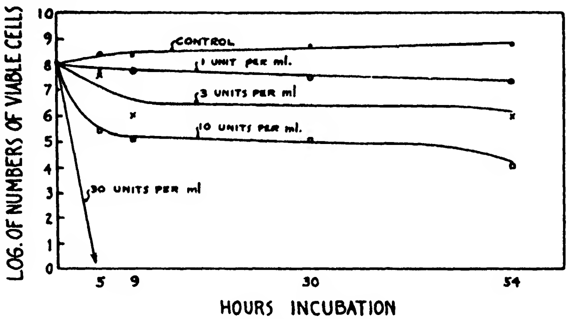

FIG. 3

Bacteriostatic and Bactericidal Action of Streptothricin

resistant colonies obtained from the various isolates. Tested under similar conditions several isolates, producing few resistant colonies, showed greatly increased numbers of resistant colonies in the course of a few more transfers. Thus, *E. coli* cultures contain cells of varying resistances to streptothricin.

Bacteriostatic vs. Bactericidal Action of Streptothricin

Some limited information on this point is already available (2), and our results confirm and extend those findings and furnish additional evidence for the presence of streptothricin-resistant cells in this particular *E. coli* culture. The curves in Fig. 3 show the logarithm of numbers of cells living after exposure for different periods of time to different concentrations of streptothricin. A massive inoculum was made so that the

medium (nutrient broth) contained 100 million cells per ml. No further significant proliferation took place. Ten units of streptothricin per ml. were insufficient to kill all the cells although 30 units killed essentially all the cells in less than 5 hours. The presence of a certain proportion of cells more resistant to streptothricin is indicated by the atypical flattening out of the death rate curves in Fig. 3. The 10 unit per ml. level reduced the number of cells from 130 million to 244,000 per ml. within 5 hours, after which the number of viable cells remained virtually constant up to 54 hours. These residual cells had such resistance that streptothricin was not lethal although their growth was prevented. The resistant cells amounted to 0.19 per cent of those originally present. Streptothricin appears to have definite bactericidal properties.

TABLE II

Inoculum Size and Streptothricin Inhibition of E. coli

Number of cells per ml.	Streptothricin units per ml. at 50 per cent inhibition
21.3×10^6	0.10
7.1×10^6	0.18
7.1×10^5	0.06
7.1×10^4	0.05
7.1×10^3	0.04
7.1×10^2	0.06
71	0.04
7	0.08

Amount of Inoculum and Streptothricin Activity

Washed cells of *E. coli* were added in different amounts to series of nutrient broth tubes containing graded amounts of streptothricin. After overnight incubation, growth was measured with an Evelyn photoelectric colorimeter. Table II shows that over a 3 million-fold range the numbers of cells are immaterial, and that essentially the same amount of streptothricin is required to obtain a 50 per cent growth inhibition starting from the various inocula. In this respect it is similar to penicillin (11, 15). The value averaged 0.076 streptothricin units per ml. to obtain 50 per cent inhibition at pH 7.0.

pH and Streptothricin Activity

The pH of the assay medium was found to be an extremely important factor governing the antibacterial activity of streptothricin. *B. subtilis* was cultivated in a series of graded concentrations of streptothricin in

nutrient broth at different pH values with shaking to prevent pellicle formation. Growth was measured turbidimetrically after 16 hours, and the inhibition curves are shown in Fig. 4. Streptothricin inhibition is strongest at alkaline reactions. From pH 5.5 to 7.0 there was no inhibition up to 0.1 unit per ml., beyond which the inhibition was rapid and complete. Inhibition began at decidedly lower levels at pH 7.5 and 8.0, requiring 0.03 and less than 0.01 units per ml., respectively. Control growth was practically equal at all the pH values. In this experiment it required 0.2, 0.07, and 0.02 units per ml. to secure 50 per cent inhibition at pH 7.0, 7.5, and 8.0, respectively. A difference between pH values of 5.5, 6.0, and 7.0 has been obtained at other times although it failed to show up in this particular experiment.

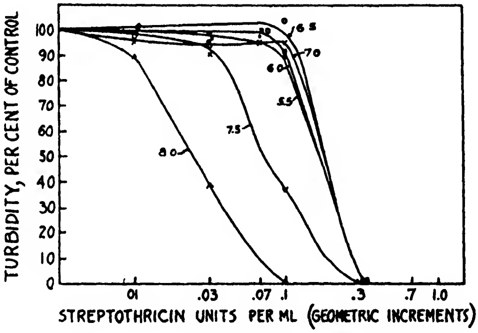

FIG. 4

pH and Streptothricin Activity

In an experiment employing *E. coli* instead of *B. subtilis*, 0.06 and 0.015 units per ml. brought about 50 per cent inhibition at pH 6.0 and 8.0, respectively. Also, an increased bactericidal effect of streptothricin was noted at the more alkaline ranges. It required more than 10 units per ml. to kill 130,000,000 *E. coli* cells within 54 hours at pH 6.0 (Fig. 3), whereas at pH 8.0, 1 unit per ml. proved bactericidal against the same number of cells within 54 hours and 3 units per ml. within 30 hours. When the pH of the agar used in the *B. subtilis* cup assay (3) was adjusted to 6.0, 7.0, and 8.0, the zones produced by a solution containing per ml. 40 units of streptothricin were 15, 21, and 25 mm., respectively, again emphasizing the critical importance of pH on streptothricin activity.

Waksman and Woodruff (1) have stated that streptothricin has the properties of an organic base. From these pH studies it appears that

streptothricin is inhibitory only when the molecule exists as the free base. When present as a salt, it is appreciably less active. Apparently the undissociated molecule is necessary for activity whether *in situ* in the cells or for membrane permeability. The reduced activity of streptothricin in the acid range is not due to its destruction since readjustment to pH 7.0 to 8.0 restores full activity. Thus, factors conducive to the existence of streptothricin as a salt *i.e.* in the dissociated state tend to reduce streptothricin activity.

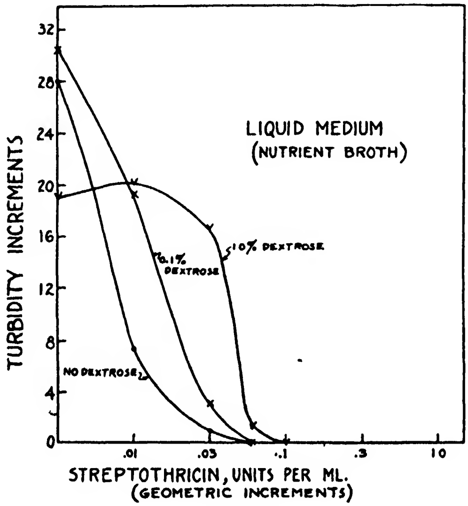

FIG. 5

Streptothricin Inhibition of *E. coli* in Dextrose Broth

Dextrose and Streptothricin Inhibition

The presence of dextrose in an assay medium (nutrient broth) appears to reduce appreciably the inhibitory action of streptothricin (Fig. 5). However, this is not due to the dextrose itself but to acid formation from it as a result of the growth (complete or pàrtial) of the test bacterium, in this case *E. coli*. This is an example of a factor conducive to the existence of streptothricin in the dissociated state, *i.e.*, as a salt. The pH values in the control tubes containing 0, 0.1, and 1.0 per cent dextrose were 7.2, 6.7, and 4.2, respectively. This last pH value inhibited further growth.

The same effect is obtained under the conditions of the cup assay for streptothricin employing *B. subtilis* (3). When 0.1 and 1.0 per cent

dextrose was present in the agar, the inhibition zones were proportionately reduced in diameter. For example, a solution of streptothricin gave inhibition zones of 18.0, 19.5, and 20.3 mm. in nutrient agar containing 1.0, 0.3, and 0 per cent dextrose, respectively. Other concentrations of streptothricin were similarly affected. The pH of the agar was measured after the zones were read and found to be 5.1, 5.8, and 7.2, respectively, further proof that the dextrose effect is due to acid formation by the bacteria and consequent existence of streptothricin as a salt. Maltose and sucrose behave similarly.

Phosphate and Streptothricin Activity. Other Salts

Studies with phosphate buffer provided further evidence of the difference in activity of the free base and salt forms of streptothricin. Nutrient broth tubes containing 0.1, 0.5, 1.0, and 2.0 per cent phosphate buffer, each at pH values of 5.5, 7.0, and 8.0, received graded levels of streptothricin and were inoculated with *E. coli*. At all pH values the phosphate had a repressive effect on streptothricin activity (Fig. 6). Thus, at pH 5.5, 10 units per ml., amounting to fifty times the streptothricin concentration required for 50 per cent inhibition in the control tubes, had no effect in the presence of 2 per cent phosphate. The inhibitory effect of streptothricin can be entirely counteracted by simply raising the concentration of phosphate to 2 per cent. This repressive action of the buffer at pH 5.5 was proportional to the buffer concentration. At pH 7.0 the repressive action of the buffer was less marked, and at pH 8.0 there was practically no difference between the curves containing buffer although streptothricin in the control tubes was definitely more active.

The behavior of streptothricin in the presence of phosphate would be expected from the idea presented above, namely, that streptothricin is less active as the salt than as the free base. The presence of an ionized salt would force the reaction in the direction of a weakly ionizable salt complex of streptothricin, in this case streptothricin phosphate. Also, under conditions where streptothricin can combine easiest with an anion to form the weakly dissociable streptothricin salt, namely, under acid conditions where streptothricin behaves as a cation, this behavior would be most marked. These results were experimentally observed (Fig. 6).

Fig. 7 proves that the phosphate effect is one of salt formation with streptothricin and is non-specific. A number of other inorganic salts and also sodium citrate had a similar repressive influence on strepto-

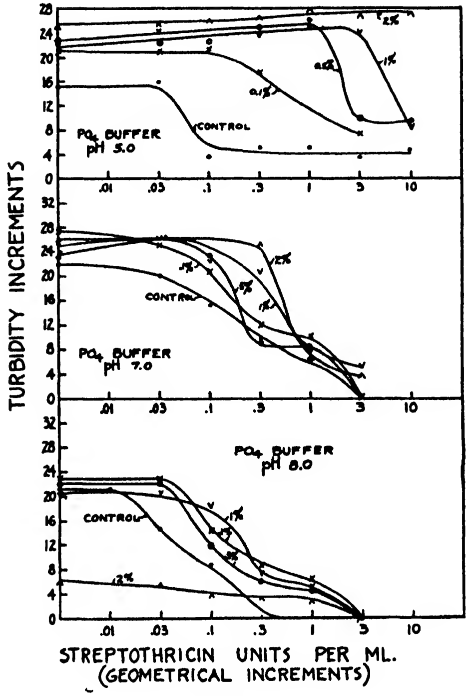

FIG. 6
Streptothricin Action and Buffer Concentration

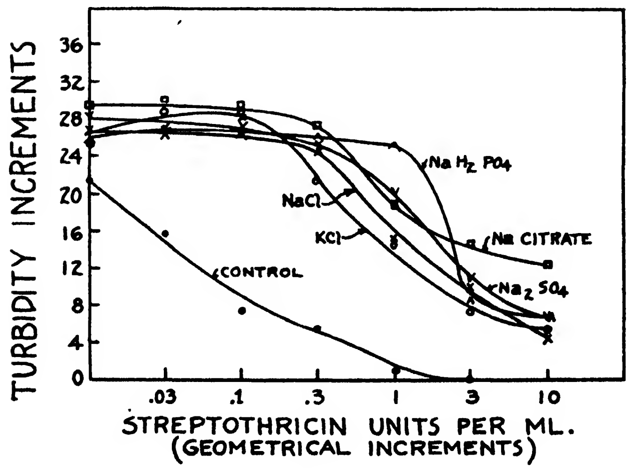

FIG. 7
Reduction of Streptothricin Activity by Different Salts

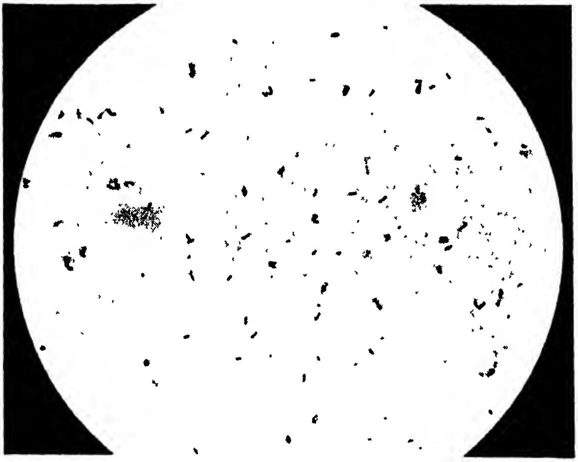

Fig. 8a
Normal Cells of *B. subtilis*

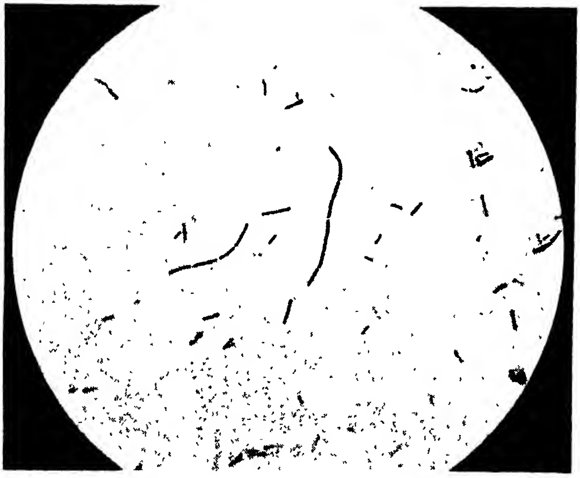

Fig. 8b
Streptothricin-Inhibited Culture of *B. subtilis*
Note the greatly enlarged individual cells and the tendency to remain unseparated so that chains from few to many cells exist

thricin action on *E. coli* in NaCl-free nutrient broth. NaCl, KCl, Na_2SO_4, and NaH_2PO_4 were used in concentrations of 1 per cent. The control curve approached the typical logarithmic curve expected for

bacterial inhibition, and if only minute traces of salts were present, the inhibition probably would be logarithmic. The plateaus typical of many of the curves obtained in the earlier experiments are now explained as being due to the presence of NaCl as a component of nutrient broth.

Streptothricin Inhibition and Morphological Changes

Cells of susceptible bacteria which have undergone partial inhibition by streptothricin are abnormally elongated. They also lose their tendency to separate and exist as long filaments, which comprise few to very many cells. These filaments appear to consist of cells which have grown to abnormally large sizes but in which the process of fission stopped short of normal. Figs. 8a and 8b show photomicrographs of normal and of streptothricin-inhibited cells of *B. subtilis*. Often there is a tendency for the filaments to remain parallel to each other in the *B. subtilis* cultures.

Similar abnormal morphological appearances have been described for bacteria under penicillin inhibition (17, 18).

Summary

The antimicrobial properties of streptothricin are of a general nature as compared to penicillin. The former is inhibitory against various organisms belonging to the bacteria, yeasts, and fungi. The latter is effective only against certain Gram-positive bacteria. The antibacterial power of streptothricin is influenced by the pH of the medium and the presence of inorganic salts. Streptothricin is inhibitory in the undissociated state, and factors tending to cause streptothricin to exist in the dissociated state reduce its activity. Bacteria subjected to streptothricin inhibition show greatly enlarged size and a tendency toward incomplete fission so that chains of cells arise.

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The Requirement of Biotin for the Growth of Pneumococci

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Received September 9, 1943

Introduction

Rane and SubbaRow (1, 2) found that they could grow highly virulent strains of Types I, II, V, and VIII pneumococci in a medium consisting of gelatin hydrolyzate, certain additional amino acids, inorganic salts, glucose, choline, nicotinic acid, pantothenic acid, and thioglycolic acid. They also obtained good growth of Types II, V, and VIII pneumococci when the gelatin hydrolyzate was replaced by amino acids. No growth was observed with Type VII pneumococci in either medium, even with the addition of other known factors.

Our problem was to grow this Type VII and other types of pneumococci on a chemically defined medium. The basal medium consisted of Labco casein hydrolyzate, salts, sugar, and other compounds. There was no growth of the test organisms on this medium unless it was supplemented by a yeast or liver extract. The properties of the unknown factor corresponded to those of biotin, and in all cases growth was obtained when the extracts were replaced by crystalline biotin methyl ester. Types I, II, V, and VIII were re-investigated and were found to require biotin for growth. This factor was probably present in the pantothenic acid concentrate used by Rane and SubbaRow.

Since the completion of this work, Landy, *et al.* (3) have predicted that Type II pneumococcus (Mellon strain) requires biotin for growth. This is in agreement with our observations. We have withheld publication of this work in the hope that we would first investigate the reasons for failure of growth of some of the organisms on this medium upon repeated transfer, but we have been unable to make this study because of the importance of some other problems in this emergency.

Experimental

Basal Medium

The basal medium was made up from two stock solutions. Solution A was heat sterilized after proper dilution and adjustment to pH 7.6. Solution B was

sterilized by Berkefeld filtration and was added to solution A under aseptic conditions.

TABLE I

Density of the Growth of Pneumococci after 18 Hours Incubation at 37° C.

Type of Pneumococci	Our Culture No.	Basal Medium	Basal Medium plus 0.0002 micrograms biotin ester per ml.	Basal Medium plus 0.1 mg. liver extract per ml.
1	1-R	0.013; 0.013	0.237; 0.237	0.284
1	1-8	0.017; 0.017	0.260; 0.284	0.108
1	1-6	0.009	0.174	0.328
2	2-8	0.027; 0.022	0.301; 0.301	0.222
2	2-7	0.009	0.260	
3	3-J	0.027; 0.022	0.301; 0.301	0.222
4	4-1	0.022; 0.017	0.244; 0.244	0.125
5	502	0.017; 0.022	0.237; 0.237	0.237
6	602A	0.022; 0.022	0.244; 0.244	0.237
7	702	0.004	0.276	0.284
7	S.V. 7	0.004; 0.004	0.237	0.215
8	801A	0.027; 0.027	0.252; 0.229	0.097
8	8-R	0.013; 0.018	0.420; 0.398	0.387; 0.377
9	9-T	0.018; 0.022	0.161; 0.155	0.215; 0.229
10	10-1	0.018; 0.013	0.125; 0.125	0.065
11	11-1	0.018	0.215	0.328
12	Cl-12	0.009; 0.000	0.292; 0.284	0.367; 0.357
13	13-1	0.027; 0.022	0.292; 0.292	0.301
14	14-1	0.013; 0.013	0.237; 0.237	0.284
15	15-1	0.027; 0.027	0.229; 0.229	0.252
18	18-1	0.013; 0.009	0.137	0.268; 0.284
19	19-1	0.018; 0.013	0.260; 0.244	0.347; 0.337
20	20-4	0.036; 0.031	0.292; 0.292	0.377
22	22-1	0.009	0.137	0.42
23	23-1	0.022; 0.027	0.244; 0.208	0.319
23	23-2	0.022; 0.022	0.237; 0.237	0.310
23	23-M	0.018; 0.018	0.222; 0.260	0.319
24	24-2	0.013; 0.013	0.187; 0.187	0.292
25	25-1	0.022; 0.022	0.174; 0.155	0.377
27	27-1	0.022; 0.027	0.125; 0.119	0.260
28	28-1	0.018; 0.018	0.086; 0.076	0.310; 0.319
29	29-1	0.027; 0.031	0.268; 0.260	0.319
33	33	0.013; 0.013	0.244; 0.252	0.222

One liter of basal medium contained 5 g. HCl hydrolyzed Labco casein, 5 g. KH_2PO_4, 250 mg. asparagine, 100 mg. *l*-cystine, 50 mg. *l*-tryptophan, 10 mg. of each of adenine, uracil, guanylic acid, glutathione, and nicotinic acid, 5 mg. choline, 2.5 mg. sodium *d*-pantothenate, and 1 mg. pyridoxin, supplied by solution

A; and 5 g. glucose, 1 g. $MgCl_2 + 6H_2O$, 0.2 mg. riboflavin, 100 mg. thioglycolic acid and 10 mg. glutamine, supplied by solution B.

Cultures

All cultures used, are part of the collection of Lederle Laboratories and were passed through mice prior to use, so as to increase their virulence.

The seed cultures from the mice were prepared on serum-peptone broth. One drop of seed culture was added to a tube containing 10 ml. basal medium plus 1 mg. of 95 per cent alcohol soluble liver. This was incubated at 37°C. for 6 to 8 hours, and 1 drop of this culture was used to inoculate each tube in the assay.

The level of the liver extract in the inoculum was so low that the amount of biotin in one drop of inoculum did not interfere with the test. Growth was for 18 hours at 37°C. Turbidity readings were made with an Evelyn photometer.

Results and Conclusions

Biotin has been found to be an essential growth factor for all 33 cultures (26 types) of pneumococci tested (Table I).

Acknowledgments

We wish to thank Dr. W. H. Peterson of the University of Wisconsin for the gift of some of Kögl's crystalline biotin ester.

We are indebted to Misses F. L. Clapp and D. Novotny and to Mrs. M. Kerr for supplying us with the seed cultures.

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On the Mechanism of Enzyme Action. Part 22
Elementary Sulfur as Hydrogen Acceptor in Dehydrogenations by Living Fusaria[1]

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Received September 6, 1943

Introduction

In the course of investigations carried out during the past twenty years, dehydrogenations served as one of the most successful means for elucidating the constitution of natural organic compounds. Significant results were first obtained by this method in the field of polyterpenes. The dehydrogenation of several derivatives of sesquiterpenes by means of elementary sulfur resulted in naphthalene hydrocarbons such as Cadalin (1) and Eudalin (2). The application of the same procedure to simple compounds, *e.g.*, limonene or terpinene, furnished *p*-cymene (2), the parent aromatic hydrocarbon of these terpenes. These reactions are carried out with carefully calculated amounts of sulfur and hydrogen sulfide evolution generally sets in at about 180°C. In so far as it is possible to ascertain, it is reasonable, however, to assume that the underlying mechanism is significantly different in catalytic dehydrogenations than in dehydrogenations carried out with sulfur or selenium.

The mechanism of intact Fusaria cell degradations is characterized by many facts: *e.g.*, they do not appear to form phosphoglyceric acid in the presence of NaF when grown on carbohydrates (3) nor do they utilize this acid to a measurable extent (4) when it is supplied as a carbon source, but are able to dehydrogenate methanol, isopropyl alcohol (Propol) (5) or ethylene glycol. Hence we were tempted to apply these preparative phenomena to enzymatic investigations and to study the rôle of elementary sulfur as a hydrogen acceptor in dehydrogenations (5a).

[1] Supported by a grant from the Rockefeller Foundation.

[2] Communication No. 32. Dedicated to Prof. G. Bredig on his 75th birthday.

There were several considerations which hinted at the possibility of the successful utilization of sulfur in these reactions. First, the low heat of formation of H_2S is + 4.8 Kcals. Second, the possibility that the H_2S so formed, due to its ionization (6), will be reoxidized by the oxygen or nitrates available in the nutrient medium partly to sulfur (a) and partly to $SO_4^=$ (b), according to the following equations:

$$S^\circ + 2H \rightarrow H_2S \tag{1}$$

$$2H_2S + O_2 \rightarrow 2S + 2H_2O \tag{2a}$$

$$2H_2O + H_2S + 4O \rightarrow SO_4^{=} + 2H_3O^+ \tag{2b}$$

Reactions represented by equations 2a and 2b may occur even though H_2S (or sulfur itself) should prove unlike cyanide (7), but similarly to yeast cells (8), to be poisonous to the respiratory system of living Fusaria to a limited extent and/or retard their growth. Third, sulfur may serve, in the presence of nitrates, as a competitive hydrogen acceptor, indirectly impeding the formation of nitrite and hydroxylamine and giving rise to a lesser amount of actually isolated pyruvic acid (PA), and accordingly, to a relatively larger amount of alcohol formed in carbohydrate fermentations (9).

We succeeded in attaining our goals by demonstrating a) that sulfur can serve as a hydrogen acceptor in enzymatic reactions, b) that the sulfur so introduced is subject to utilization according to equations (1) and (2a), and c) an additional enzymatic reaction operative with Fusaria.

Experimental

The enzyme material employed was the same as in previous (9) work. However, in quantitative experiments Fusarium lini Bolley (FlB) No. 5140 from the Biologische Reichsanstalt, Berlin-Dahlem, was exclusively used. Stock cultures of the organisms were maintained on the following nutrient medium:

20.00 g. Glucose
1.00 g. Potassium nitrate
1.50 g. Potassium dihydrogen phosphate
0.75 g. Magnesium sulfate, hydrated
Water to 1 liter.

The cultures were transferred at intervals of two weeks and periodically examined to check their purity. The nutrient medium used during the experiments was the following:

5.00 g. Potassium nitrate
5.00 g. Potassium dihydrogen phosphate
0.75 g. Magnesium sulfate, hydrated
Water to 1 liter.

The initial pH was 4.4. All chemicals used were of T. P. grade.

Procedure

Three liter Fernbach flasks, as in earlier investigations from this laboratory (10), proved of great advantage for this type of experiments. When glucose or glycerol were the carbon source, they were added to the medium before sterilization. Propol was introduced after sterilization of the salt solution. Inoculations with the organism were made by means of a spore suspension obtained from glucose-agar. Sulfur was added on the day desired, usually the fourth, in colloidal form with sterile technique. Dispersions of 1 mg. per ml. of water or solutions of crystalline sulfur (1 mg. per ml. of Propol) were used.

Analytical methods

The hydrogen sulfide evolved was detected with the highly sensitive lead acetate paper strips. Sulfate was precipitated by barium chloride after treatment of the medium with trichloroacetic acid and acidification with HCl. Propol was analyzed by the procedure previously (5) described. Glucose determinations were carried out polarimetrically. Alcohol was oxidized by potassium dichromate, and pyruvic acid (PA) was precipitated with 2,4-dinitro-phenylhydrazine (9). Mat weights were determined by filtering the mycelium through tarred porous alundum crucibles and washed with distilled water. Drying was carried out in an oven at 60°C. until constancy was obtained.

Results and Discussion

Preliminary examination showed that when sulfur was added to different species of Fusaria grown on a medium having Propol as the carbon source, a positive test for hydrogen sulfide was obtained in less than 15 minutes. It was evident that sulfur was acting as a hydrogen acceptor, Propol being the hydrogen donor. The acid nature of the medium, which contained potassium nitrate, and the instability of H_2S, increased under our experimental conditions, prompts us to assume that part of the H_2S escaped, some was transformed to $SO_4^{=}$, and some reoxidized to sulfur. Accordingly, it would appear that we are justified in postulating a sulfur cycle in which the rate of reoxidation to sulfur or $SO_4^{=}$ is surpassed by that of H_2S formation. The formation of sulfate was proven by experiments in which the magnesium sulfate of the nutrient medium was replaced by magnesium nitrate and elementary sulfur. Larger quantities (250, 500, 10,000, and 50,000 γ) of sulfur were added to the inoculated medium starting from the zero day. Positive sulfate tests were obtained from time to time in the course of a three weeks long experiment.[3]

[3] However, the agar-containing spore suspension itself did give rise to traces of sulfate ion (11) formation in the nutrient medium.

Dehydrogenation of Propol and Glycerol in the Presence of Sulfur

Quantitative experiments on the dehydrogenation of Propol tend to show an increase in the rate of disappearance of the substrate when the growth of the organism, evaluated on the basis of mat weights, is taken into consideration. For example: after administration of 1,000 γ sulfur, the mat weight determined on the 14th day, shows a decrease of 56%, as compared with a decrease in dehydrogenation of only about 9%.

TABLE I

Effect of Sulfur[2] on Dehydrogenation of Isopropyl Alchol[1]

Day of Analysis		blank	blank	25	50	500		1000		2000	5000	10000		25000	50000
								gamma							
0	0	183.6	188.5	183.7	186.6	186.9	187.5	190.9	189.0	186.7	185.1	187.6	189.0	183.4	191.1
	6		32.8				36.9		31.7				30.6		
7		32.8		28.0	33.2	36.2		31.8		30.9	25.9	23.1		26.3	14.8
	8		47.4				50.9		48.7				44.9		
10		59.4		62.6	57.1	52.3		52.8		44.3	46.8	32.0		46.4	31.8
13	13	81.4	122.5	78.3	81.7	79.6	122.3	80.4	80.8	71.6	67.7	65.7	91.4	61.8	50.7
	14		127.7				125.3		117.4				112.1		
17		129.4		129.3	126.6	125.1		113.9		91.9	93.0	84.9		87.0	76.7
								Mycelium Weights							
	14		166				87		67				73		
17		112		95	78	65		38		33	38	18		23.0	18

[1] Amount in milligrams disappearing per 50 ml. of nutrient medium.

[2] Concentration of sulfur is expressed in gamma per 700 ml. Sulfur was added on the fourth day of growth. FIB was used.

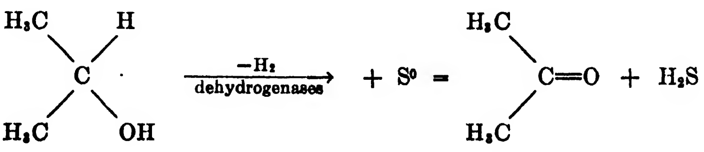
TABLE II

Effect of Sulfur on the Growth with Glycerol as a Carbon Source

	Added on 0 day		Added on 4th day	
Blank	5000 γ	3000 γ	3000 γ	1000 γ
1185	2179	1267	1923	1239

In another case, using 25,000 γ sulfur, the decrease in mat weight on the 17th day amounted to 90% as compared with the blank, and in comparison with a decrease in dehydrogenation of about 32%.

The following equation may picture in a simplified way the course of this reaction:



In utilizing glycerol, which was an excellent carbon source for dehydrogenations (12), its superiority, as compared with the strongly inhibitory Propol, served to overcome the detrimental effects of sulfur, H_2S, or both on the growth of the organism, as can be easily seen from the data recorded in Table II.

Growth was better, as compared with the blank, even after adding sulfur simultaneously with inoculation.

The Function of Sulfur in the Course of Alcoholic Fermentation of d-Glucose and d-Xylose

Applying sulfur in the course of alcoholic fermentation of carbohydrates, it was possible to make decisive observations as to its rôle in

TABLE III

Effect of Sulfur on Glucose Fermentation

	Blank			500 γ			1000 γ			10000 γ		
Day	Glucose	P.A.	Alcohol	Glucose	P.A.	Alcohol	Glucose	P.A.	Alcohol	Glucose	P.A.	Alcohol
	g.	*mg.*	*mg.*	*g.*	*mg.*	*mg.*	*g.*	*mg.*	*mg.*	*g.*	*mg.*	*mg.*
0	4.03			4.01			4.01			4.00		
7	1.43	128	442	1.53	127	451	1.63	127	490	1.59	104	504
10	3.26	159	1052	3.23	151	1049	3.53	141	1136	3.42	108	1150
Mat weights												
	1529 mg.			1570 mg.			1935 mg.			1803 mg.		
Thiamine values												
	21.7 γ/g.						21.5 γ/g.			20 γ/g.		

Glucose expressed in grams per 100 ml.
P.A. and alcohol expressed in mgs. per 100 ml.
Sulfur in gamma per 700 ml.

our previously (9) postulated phase sequence. From the data recorded in Table III it can be seen that the presence of sulfur brings about an increase in the disappearance of glucose as compared with the blank. The intermediary key product in fermentations, pyruvic acid, accumulates to a lesser extent in the experiments with sulfur and decreases proportionally with the quantity of sulfur added. Accordingly, and as is to be expected, the mat weights and the amounts of alcohol produced are increased. This would indicate that some of the hydrogen freed by the dehydrogenases, and acceptable either by oxygen, nitrates, or some intermediates present, is diverted to the elementary sulfur available, which is taking a competitive part in the chain of reactions. Hence, a lesser

amount of nitrate is reduced to nitrite, and the inhibition of carboxylase becomes less predominant. In turn, the isolable part of the total amount of pyruvic acid becomes diminished. Evolution of H_2S was established in both glucose and xylose fermentations. In connection with earlier findings (10), our observations seem to signify also, that in distinction to yeast, the 'cyanide-stable' or metal-free respiration in Fusaria accounts for a much larger extent of the oxygen consumption than the cyanide-sensitive part of the enzyme system.

The amount of thiamine[4] synthesized in the presence of elementary sulfur remained unchanged, in accordance with observations made with certain torulae (13), when its single components were administered during the course of fermentation.

Our results again corroborate the fact that there may be too much importance attached to the phospho-organic esters as overall intermediary transfer agents, and that all biochemical reactions do not follow similar general pathways.

Acknowledgment

The authors are under obligation to Dr. J. C. Wirth of this laboratory, for his criticisms and continuous encouragement during the progress of these observations.

Summary

1. Elementary sulfur serves as a useful hydrogen acceptor in dehydrogenations and alcoholic fermentation of hexoses or pentoses by Fusaria.

2. The elementary sulfur present is converted to H_2S, which, in turn, is partly reoxidized to sulfur, and partly to $SO_4^{=}$.

3. The demonstration of the participation of elementary sulfur in the alcoholic fermentation of carbohydrates serves as additional support to the phase sequence earlier presented for these processes.

4. In agreement with the effect of cyanide on Fusaria, no measurable inhibition of their respiratory system by H_2S was observed.

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[4] The authors' indebtedness is expressed to Mr. M. Soodak, for his courtesy in carrying out the thiamine determinations.

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BOOK REVIEWS

Chemistry and Methods of Enzymes. By James B. Sumner and G. Fred Somers. Academic Press, Inc., Publishers, New York, N. Y., 1943. xi + 365 pp. Price \$5.00.

Of great importance to all biochemists and biologists is this book which offers so much specialized information in a small volume. The authors begin with a compilation of what they consider the most important dates in enzyme history. Many enzymologists will disagree with the opinion of the authors but it is, of course, impossible for such a list to meet general approval. Those who have occasion to review the literature on any enzyme will doubtless find most of the important references in the bibliographies which follow each chapter.

The enzymes are dealt with in the following classifications: esterases, carbohydrases, enzymes of carbohydrate metabolism, nucleases, amidases, proteolytic enzymes, oxidases, nuclein desaminases, desmolases, hydrases and mutases. Almost half of the book is devoted to the oxidative enzymes which are discussed in detail under the headings: the iron enzymes, the copper enzymes, dehydrogenases containing coenzymes I and II, oxidases which transfer hydrogen to cytochrome, the yellow enzymes, nuclein desaminases, desmolases, and miscellaneous oxidases.

The style of the book is particularly well-adapted for its use as a text. Each enzyme is discussed under such headings as *Historical, Occurrence, Action, Specificity, Method of Determination, Activation and Inactivation*, and *Preparation.* Such an arrangement makes for clarity but, in the cases in which the known facts are limited, much space is wasted. The liberal use of tables, figures, illustrative equations and formulas makes it excellent collateral reading for all biochemistry students. Enzyme research is so largely an art that the enzyme chemist gains a great deal from a general knowledge of empirical procedures successfully employed with enzymes out of his field. Thus the authors are fully justified in devoting such a large proportion of the book to methods. Indeed, a chapter or two devoted to the physical chemistry applicable to enzymes (and proteins) would have been greatly welcomed. The solubility criterion of purity, electrophoresis, ultracentrifugation, osmotic pressure and diffusion rate determinations, denaturation, and monomolecular films are cited as subjects which deserve more than passing attention, especially in a book which carries "methods" in its title.

The mode of presentation is concise without being abrupt. There seem to be few mistakes in the context, but some have managed to slip by both authors and editors. One such occurs on page 58 where phytin is preferred to as the K, Mg salt of inositol hexaphosphate (instead of the Ca, Mg salt). In the second paragraph on page 139, there is apparently an omission which leads to a startling conclusion. On page 158, the classification of the enzymes occurring in papaya latex is misleading, since Bergmann's classification and Balls' classification are independent

of each other. The statement (on page 159) that ficin is not activated by cysteine, hydrogen sulfide, or hydrocyanic acid is incorrect. Another error occurs on page 229 in the section on plant glutamic acid dehydrogenase, in which the statement is made that coenzyme I is required for the action of this enzyme (even though the system was found to react with molecular oxygen). The data in the original article do not support this claim. Such minor details do not detract from the value of the book as a useful tool for the biochemist concerned with enzyme problems. The book is quite up-to-date and the reader must bear in mind that some of the conclusions presented are controversial and may be subject to revision.

Most enzyme chemists in the United States have felt that a modern book in the English language devoted to enzymes was very much needed. Hence it is an event of considerable significance when such an excellent volume appears.

BERNARD AXELROD AND MARIAN W. KIES, Albany, Cal.

The Endocrine Function of Iodine. By WILLIAM THOMAS SALTER, Assistant Professor of Medicine, Harvard Medical School, Associate Physician, Thorndike Memorial Laboratory, Boston City Hospital. Harvard University Press, Cambridge, Massachusetts, 1940. xviii + 351 pages. Price $3.50.

This book contains eleven chapters, an appendix and a good index. Each chapter is the result of a well digested survey of the literature on one of the following topics: iodine balance and endocrine balance; iodine stores in body tissues; iodine compounds of biological importance, circulating iodine, thyroid activity; endocrine balance; iodine and the pituitary-ovarian axis; neurological influence; iodine balance; radioactive iodine; clinical problems. The appendix, which is for laboratory workers, contains detailed directions for the determination of iodine in amounts from a fraction of a microgram up to several milligrams. Methods are given for the partition of the proteins of the thyroid gland and of the blood plasma into thyroxine-like and diiodotyrosine-like fractions, and for the fractionation of the plasma into water soluble iodides and organic iodine compounds bound to protein. There is also a description of a method for the assay of thyrotropic hormone.

To investigators in the field under discussion it is apparent that Doctor Salter has drawn a wide circle around the available information and after mature deliberation he has clearly indicated his interpretation of the experimental results, or else he has stated: "... this chapter must remain a simple record of present quandaries and obscurities."

The labor involved in the preparation of the volume is obvious. There are 588 references and 100 incidental references. The segregation of this number of investigations into separate topics which are treated as individual subjects had necessitated a certain amount of repetition, but the result achieved should be a source of satisfaction to the Editorial Committee of Harvard University Monographs in Medicine and Public Health. This volume is Number 1 and it establishes a high level of attainment as a precedent for future members of the series.

In the interpretation of the experimental results Doctor Salter was confronted with data which were in some cases flatly contradictory, and in many instances acceptance of one viewpoint has meant rejection of the results of other extensive investigations. However, in the conclusions which have been reached, it is the

opinion of this reviewer that Doctor Salter has remained on firm ground and that if this volume is revised after some years but little will have to be changed. Perhaps this is high praise and may seem to indicate that it is believed that final and complete answers have been secured to all the many questions. The whole story, however, has not yet been told and much work will have to be carried out to fill in the gaps which are clearly apparent.

The preparation of this volume at the present time has permitted the inclusion of work which answers some questions of long standing, such as the in vitro synthesis of thyroxine from diiodotyrosine, but it has been written just too soon to include other still more recent work which promises to be of great importance both from the theoretical and practical viewpoints. One such new contribution is the specific effect of thiouricil on the synthesis of thyroxine in vivo. But whatever new and unforeseen results are obtained they can probably be fitted into the general scheme of Doctor Salter's book without serious conflict or flat contradiction.

Doctor Salter has adopted a tolerant and generous attitude toward the published work on the iodine content in the body fluids. His position is stated by this footnote: "As pointed out in the text, no great importance is attached to the absolute magnitude of figures given for blood iodine concentration except in a few cases where the absolute truth is ostentatiously discussed. In most physiological problems, only relative values, contrasted with a normal control, are needed. In evaluating statements involving iodine concentration, therefore, the reader must become accustomed to thinking in terms of a sliding scale. In nearly every case where iodine concentration is mentioned, the normal control figure is given simultaneously. At the present stage of technical facilities for determining iodine in minute amounts it is necessary to think in terms of relative truth without insisting upon absolute truth." At the present time no other course is possible but it is obvious that controversies will continue until adequate analytical procedures are available.

In the blood, iodine occurs as iodide ion and also in organic combination. Two methods have been used to fractionate the iodine-containing compounds. The first is to coagulate the protein with heat and acetic acid and carry down the organically bound iodine as an integral part of the protein. The precipitate contains the P (protein) and the solution contains the I (inorganic iodine compounds). The second method is to use an organic solvent, as alcohol, to precipitate the iodine compounds combined with protein. The precipitate is designated organic iodine and the compounds soluble in alcohol are designated inorganic iodide. Doctor Salter has chosen the first method as more accurate, and on this point this reviewer is in agreement. Whether the further fractionation of the P fraction into T (thyroxine-like) and D (diiodotyrosine-like) components can furnish results which are significant remains to be shown.

Two questions concerning the thyroid gland are now of several years' standing. One is the synthesis of thyroxine in vivo and the other is the preparation of material which possesses typical thyroxine-like activity through the coagulation of peptides previously hydrolysed from proteins of the thyroid gland. This results in a plastein through the action of pepsin. Doctor Salter has included both of these subjects in his book but no final proof which answers all questions is given.

For the synthesis of thyroxine in vivo he resorts to the question: "Does thyro-

nine (desiodo thyroxine) exist preformed in serum protein as an essential amino acid awaiting iodination?" The recent synthesis of thyroxine from diiodotyrosine in vitro and the possible mechanism for this synthesis suggested by Westerfeld which has appeared after Doctor Salter's book was published would indicate that the answer to Doctor Salter's question is no, and that thyroxine is synthesized only by the coupling of two molecules of diiodotyrosine, with elimination of one of the side chains.

The formation of a plastein which possesses typical thyroxine-like activity through the coagulation of peptides with pepsin may be explained in one of two possible ways. First, the peptides supposed to contain only diiodotyrosine actually contain thyroxine, the action of pepsin results in the formation of large aggregates which include thyroxine, and when this material is administered to the patient with myxedema the small amount of thyroxine which was originally unable to exert demonstrable activity produced sufficient influence on the basal metabolic rate so that its characteristic behavior could be measured in quantitative terms. The second alternative is that under the influence of pepsin diiodotyrosine was induced to couple with itself with the formation of thyroxine. The first possibility could be ruled out if it could be shown that no trace of thyroxine was present in the peptide before coagulation with pepsin. This has been shown physiologically by demonstration that the peptide fraction did not influence the basal metabolic rate. However, this is not chemical evidence that thyroxine is not present and until chemical proof is forthcoming that no trace of thyroxine is present in the peptide fraction before coagulation with pepsin, the most probable explanation for the creation of thyroxine-like activity through the action of pepsin is through the formation of the plastein which has a high molecular weight and is the carrier of the prosthetic group, thyroxine. Nowhere in Doctor Salter's volume is there evidence that diiodotyrosine under any condition can produce the characteristic physiologic effects of thyroxine and there is no evidence to indicate that the combination of diiodotyrosine and thyroxine is more active than thyroxine itself. There are many results which clearly show that the physiologic effect of thyroxine is greatly enhanced when it is combined with protein.

There are a few errors in the book: Page 154, line 24, "decrease" should read "increase." On page 197, third line from the bottom, "negative" should read "positive." Page 220, line 11, the experimental animal used is not given. It was apparently the rabbit. Page 243, line 6 and page 246, line 11: The basal metabolic rate was normal, not zero. Page 276, line 21, the solution contained potassium carbonate and presumably sulfite, not sulfuric acid. Finally, there does not seem to be any advantage in the use of the word "adrenine" for adrenaline. On page 179, paragraph 2, both words are used for the same substance. It is highly probable that everyone who reads the volume will recognize the word adrenaline; whether everyone will know that adrenine is a synonym is open to question.

The book is printed on good paper with large type and the charts and tables are well reproduced.

E. C. KENDALL, Rochester, Minnesota

Advances in Enzymology and Related Subjects of Biochemistry, Volume III, edited by F. F. NORD AND C. H. WERKMAN. Interscience Publishers, Inc., New York, 1943. 408 pp. Price $5.50.

Volume III of the Advances in Enzymology again fulfills the expectations of the biochemists and investigators in related fields. This volume has a special character in that it commemorates the death of Richard Willstätter. An excellent photograph and a few remarks by Arthur Stoll magnificently describe this eminent genius to whom enzyme chemistry owes so much.

A large variety of subjects is discussed, and the editors are to be congratulated upon their successfully enlisting the coöperation of outstanding authors. Not only do these articles offer complete information on the most important advance, but more valuable is the fact that this collection provides a possibility for extensive discussion, historical consideration, outlook of future work, and disgression into neighboring fields, for which there is too limited opportunity in the periodicals covering this field. Thus, a well-rounded picture is developed for the reader.

The field of carbohydrate metabolism is most extensively treated in two articles, one by E. S. Guzman Barron on "*Mechanisms of Carbohydrate Metabolism. An Essay on Comparative Biochemistry*," and the other by H. A. Krebs on "*The Intermediary Stages in the Biological Oxidation of Carbohydrate.*" Review articles on this subject are very numerous, and, as a rule, very uniform. A pleasant surprise is Barron's way of presentation. It is delightful to follow his discussion from the first to the last page. Emphasis is laid on the multiplicity of pathways of carbohydrate metabolism, and the author raises his voice against unjustified simplification by some investigators. This contribution certainly is more than an essay. H. A. Krebs' interesting article is centered on those phases of carbohydrate metabolism in which he has contributed so much himself. The discussion will be found exciting by those active in the same field. It is timely that Krebs has taken the opportunity to defend his much discussed "citric acid cycle." Just how well he succeeds the reader will have to decide.

Irvin W. Sizer discusses "*Effects of Temperature on Enzyme Kinetics*," in a chapter which could be part of an extensive textbook of enzymology. Since such a book does not exist, his contribution is well placed in the "Advances" and will be found very helpful to the specialist. W. T. Astbury reports on "*X-Rays and the Stoichiometry of the Proteins.*" It is impressive to be reminded what a dominant role the methods of physical chemistry are playing in the biochemistry of proteins. The author has succeeded in making his presentation understandable even to the reader with moderate knowledge of physics.

A. E. Mirsky reviews "*Chromosomes and Nucleoproteins.*" Comprehensive articles on this subject are scarce, and there has been great demand for such a treatise. It will help toward further coöperation and reciprocal understanding between chemists and cellular physiologists who have been going separate ways for such a long time. The picture that Mirsky develops is unusually attractive. On the same high level is Kurt H. Meyer's report on "*The Chemistry of Glycogen.*"

Kjell Agner describes an enzyme of peroxidase character which he has isolated recently from empyemic fluid. He calls it "*Verdoperoxidase.*" This paper represents a report of original research. Although interesting, it might as well have been published in a periodical. Jesse P. Greenstein has contributed a review on "*Recent Progress in Tumor Enzymology.*" Despite very extensive investigations and admirable endeavor progress is restricted mainly to collecting data on a multitude of enzymes. A generally applicable biochemical test serving as "a window for the diagnosis of malignant growth" is still lacking.

The technology of enzymes is represented by an article of W. V. Cruess on *"The Role of Microorganisms and Enzymes in Wine Making."* In his stimulating essay the author demonstrates what an elaborate art wine making is. One wonders how our ancestors could arrive at a palatable drink without all that information. Or, are present day wines better?

Two vitamins are covered by excellent reviews: *"The Chemistry and Biochemistry of Pantothenic Acid"* by Roger J. Williams, and *"The Chemistry and Biochemistry of Biotin"* by Klaus Hofmann.

F. Schlenk, Galveston, Texas

The Dynamic State of Body Constituents. By Rudolf Schoenheimer, M.D., Late Associate Professor of Biological Chemistry, Columbia University, Cambridge, Massachusetts, Harvard University Press, 1942.

The strong influence of Rudolf Schoenheimer's work on the shaping of present-day biochemical thinking may be attributed equally to the discovery of new facts and to imaginative interpretation. These three lectures, written shortly before his tragic death and not entirely finished, reflect in their sometimes almost note-like form directly and sincerely his contemplative attitude. They are like an overture to new biochemistry. In the two first lectures the work on the behaviour in the body of fats and proteins is recounted. The last chapter deals with the emergence of excretory products. Particularly this last chapter, as does isotope work in general, furnishes numerous examples of comforting agreement between *in vivo* and *in vitro* experimentation: Transamination, transmethylation, creatine and urea syntheses are the most important instances.

Against the background of isotope analysis throughout this essay the artificiality is stressed of interpretations built on notions which are borrowed from man-made chemistry. All the components of an organism, it is explained, are in a state of continuous rebuilding. Living matter constituted by metastable systems is in constant need of rejuvenation. Without this it would deteriorate into dead matter, and it is to counteract the chemical deterioration that maintenance of life requires the influx of energy, vic. metabolically generated energy. With the discovery of a turnover in the constituent units, now metabolism is explained more satisfyingly as a characteristic of anything actively alive.

The term *dynamic state* is applied to express aliveness of the body matter. Essentially it stands for *steady state*, supplanting the somewhat colorless "steady" by the more expressive "dynamic". A development of the theory of the steady, or dynamic, state, applied penetratingly to highly complex systems, seems desirable. It appears that in a living system the mere description of a participant by chemical constitution is insufficient. This has to be supplemented by its steady state characteristics, such as half-life time, mechanics of regeneration and so forth. Only through all these functions together is a constituent fully tied into an organismic whole.

Unlike spontaneous equilibrium, a steady state is maintained through supply of energy. The ubiquity in living systems of surplus energy indicates a superposed metabolic, a "second order" chemistry, built upon and maintaining chemical individuals. With clearer recognition of an infiltration of structure by

metabolism biological chemistry tends to develop more and more into a chemical biology.

FRITZ LIPMANN, Boston, Massachusetts

Proteins, Amino Acids and Peptides as Ions and Dipolar Ions. By EDWIN J. COHN AND JOHN T. EDSALL, 686 pages, Reinhold Publishing Corporation, New York, N. Y., 1943. Price $13.50.

There are a variety of approaches to the problems presented to scientists by that comprehensive group of substances that are entitled proteins. Fischer's researches in the first decade of the present century typify the early attack on the problem from the traditional standpoint of the organic chemist which demonstrated the amino-acid constituents and identified the larger hydrolytic split products, the polypeptides. The large size of such molecules and the numerous constituent amino-acids implied that classical synthetic methods would not be too helpful in the elucidation of protein structures. New tools would be needed, new techniques would require development. The present volume reveals something of the scope of these tools and techniques. As the title page indicates it is concerned largely with these materials as ions and dipolar ions, so that it is, therefore, the physico-chemical aspects of the subject that are stressed in the volume.

The techniques comprise spectroscopy both in the infra-red and ultraviolet, X-ray diffraction, centrifugal studies of diffusion and sedimentation, viscosity and double refraction of flow, dielectric constants and dipole moments of dipolar ions and of protein solutions, together with the physico-chemical techniques pertaining to these substances as dipolar ions such as acid-base equilibria, molal volumes, heat capacity, compressibility, solubility, interactions of ions, dipolar ions and proteins associated so intimately with the name of the senior author. In each case the attempt is made to incorporate in the treatment the fundamental theoretical principles upon which the application of the techniques to the problem are based. For this reason Professor Scatchard, for example, provides in 50 pages of Chapter 2 the fundamentals of thermodynamics that are needed beyond one's elementary preparation in this subject, in order to cope with the more complex problems of equilibria that these systems entail. Professor Kirkwood summarizes the theory of interactions between ions and dipolar ions in a final chapter of Part I of the book. The theory of electrophoretic migration, which underlies the quantitative analytical methods developed by Tiselius, Thorell, Longsworth and MacInnes which have become so important a practical aspect of protein science, is detailed by Dr. Hans Mueller. Dr. Oncley contributes the theory, methods and results of dielectric constant research. Within a single volume, therefore, the reader has not only a summary of a large body of physico-chemical research but also authoritative outlines of its theoretical background. With so broad a scope of field the task was beyond that of two authors and, hence, the co-operation of distinguished collaborators.

Part I of the book deals with the simple dipolar ions whose structures are exactly known. But, as the authors themselves point out, valuable as this material is and necessary, also, to the study of the proteins themselves, the protein problem is vastly more complex than that of the simple molecules there treated.

Not only is the dipolar character of proteins important, but, it emerges that the components other than the amino-acid peptide structure, prosthetic groups including carbohydrates, heme derivatives, phospholipids, carotenoids and the combinations of nucleic acids and amino-acid residues comprising the nucleoproteins may have a determining rôle in the properties of particular proteins. The enzymic activity of proteinases specific to particular protein configurations is one evidence of the controlling aspects of molecular architecture in protein behavior. A particular protein, for example, horse haemoglobin, provides an illustration of how many different techniques can be employed to provide only a very partial solution of the problems involved. Acid and base combining capacity, amino acid constituents, molecular weight, magnetic characteristics of oxy- and reduced haemoglobin, while they have advanced our knowledge, still leave us far from our goals. This volume reveals what a wealth of experimental effort can be devoted to progress in this particular field and what a high degree of technical scientific equipment is possessed by those who are forwarding this effort. These techniques have given us the molecular concept of the proteins, have given us their coarse structure, fibrous or globular, their shape factors, with an ever-increasing refinement, the present status of which this volume decisively reveals and makes readily accessible to the reader.

There are definite limitations to the treatment of proteins in this volume which the authors themselves point out and others which will be obvious to the reader. Practically all discussion of protein surface films has been omitted and there are only brief references to the important problem of denaturation. Indeed, one might observe that, from the point of view of what chemical kinetics can teach with respect to protein systems, the book falls short of the standards that have been maintained with respect to the thermodynamics of protein systems and their behavior as individual entities to various physical factors imposed upon them. Another equally fascinating volume on proteins remains to be written in which the important topics will be the influence of temperature and pressure on the changes and velocity of change that occur in protein systems, the specific interactions, chemical in nature, that added materials may cause. These chemical and kinetic factors have their contribution to make to the elucidation of protein structure and behavior. The present volume is in the mood of Sir W. B. Hardy, Jacques Loeb, T. B. Osborne and S. P. L. Sorensen, to whom it is dedicated. It marks a milestone of research development in the science of proteins, but there await the younger scientists in the post-war era rich areas of terrain to explore.

Hugh S. Taylor, Princeton, New Jersey

The Blood Picture in Furunculosis Induced by Bacterium salmonicida in Fish*

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Introduction

"Furunculosis" is a highly fatal epizootic disease occurring in a wide variety of fish throughout the world. An organism isolated in 1894 (1) from fish infected with this disease was named *Bacterium salmonicida*, Lehmann and Neumann (2). In 1909, Plehn (3) fully established the organism as the causative agent of the disease. The bacteria have been encountered in both fresh and salt water species during widespread devastating epidemics in Switzerland, Germany, France, Great Britain, the United States, and Japan (4; 5, page 437).

Although the world-wide economic losses attributable to "Furunculosis" are difficult to evaluate, the disease has been held directly responsible for serious restrictions to fishing in the waters of Great Britain, where, as in other bodies of water, the disease resides endemically (4, 6, 7).

Published investigations deal to a limited extent only with the causative organism and the gross pathology observed in infected fish. In brief, "Furunculosis" is characterized by a generalized septicemia wherein the organism localizes in muscle tissue and where it forms visible ulcerous areas of hemorrhagic necrosis.[2] Moreover, certain organs as

* Published with the approval of the Director of the Wisconsin Agricultural Experiment station.

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[2] The necrotic areas readily perforate externally; when arising naturally they appear as small ulcers on the skin, discharging a red, pus-like material consisting of bacteria, blood corpuscles, and disintegrated muscle tissue. These ulcerous lesions typify what is commonly known as the "Red Spot Disease."

the liver, kidney, and spleen are especially subject to liquefactive degeneration. The proteolytic nature of *Bacterium salmonicida* (4, 5) has served to explain tissue liquefaction observed in diseased animals. Accumulated experimental observations are described more fully in two older works (4, 5).

The absence of any prophylactic or curative treatment for this contagious disease leaves the immediate destruction of all infected, as well as exposed, fish as the only control measure (8). Since we were unaware of any published studies on the chemical changes induced by the disease in experimental animals, it was felt that a biochemical survey might provide some information which could be used to combat the infection. This report describes initial observations on the changing blood picture in carp artificially inoculated with *Bacterium salmonicida.*

Experimental

The present study consists of two separate but similar series of experiments which were performed during the spring months at the biological laboratories of the James Nevin State Hatchery, Madison.[3] Earlier studies on the blood of fish (9) suggested that the carp, *Cyprinus carpio*, was an ideal experimental animal where repeated handling was necessary in taking consecutive blood samples; thus 1 to 2 year old carp were used. Fifteen of the test animals, chosen at random, were cultured at the beginning of each experiment and were found to be free of *Bacterium salmonicida.* Twenty-four animals were inoculated in the first series, and 36 in the second. Two fish were placed in each of a double row of glass aquaria. Each aquarium received an individual supply of filtered hatchery spring water which was maintained at a constant temperature of 6–8° C. In the first series, one uninoculated control animal was placed in each tank together with an inoculated specimen; in the second series, control animals were maintained in separate tanks.

The strains of *Bacterium salmonicida* were originally isolated from diseased carp (10). Six such strains were used. The fish were inoculated by injecting a suspension of the organisms into the abdominal cavity at the base of the pelvic fin. The organisms were grown on fish infusion agar (10) slopes for 48 hours at 20°C. After 2 ml. of sterile physiological saline solution had been added to the slope, the organisms were carefully scraped from the agar with a sterile inoculating needle. When the

[3] This project was made possible by the cooperation of the Biology Division of the Wisconsin Conservation Department and its Chief, Dr. Edward Schneberger.

bacteria had been evenly suspended in the saline, 1 ml. of the suspension was drawn aseptically into a sterile hypodermic syringe. The suspension was immediately injected into the fish and recorded as $\frac{1}{2}$ the amount of a 48 hour slope culture. One ml. of sterile saline solution was again added to the same agar slope tube, the bacteria were resuspended and 1 ml. was drawn off and injected; this was recorded as $\frac{1}{4}$ of the 48 hour slope culture. The procedure of diluting the culture serially was continued until $\frac{1}{24}$ the culture of each strain was obtained for the first series of injections. In the second series, the dilutions were extended to $\frac{1}{32}$ and $\frac{1}{64}$ of the culture from each strain of *B. salmonicida* used.

At death, all animals were examined for the presence of *B. salmonicida*. On opening the peritoneum a hot sterile inoculating needle was thrust into the kidney and allowed to cool. Then the needle was used to streak some of the kidney substance on a fish infusion agar slant which was then incubated for 7 days at 20° C. The development of a characteristic brown pigmentation on the slant cultures indicated the presence of the organisms. The technique described resulted in obtaining 90 to 100 per cent of pure cultures from infected fish.

In preparation for sampling, the fish were placed under light anesthesia by the electric shock technique previously described (9). Blood samples were taken by heart puncture into a syringe containing 0.1 *M* sodium oxalate solution. Samples requiring plasma analyses were centrifuged immediately and blood counts were also performed at once. The following determinations were made: red and white blood cell counts, total plasma protein and individual constituents, albumin, globulin, and fibrinogen, blood sugar, and analyses of the non-protein blood filtrate; total nitrogen, amino acids, urea, uric acid, creatine, and creatinine. The treatment of the blood and methods of analyses were the same as used in other studies on normal fish (9).

Results

Cultures from the sixty inoculated fish were always positive for *Bacterium salmonicida* with but two exceptions. Likewise, uninjected fish which had been placed in the same aquaria with the inoculated fish exhibited the organisms on culturing. Although, the first control fish thus infected, did not die until after the last of the injected ones had died, uninjected control animals placed in separate aquaria did not contract the disease. There was no correlation between the amount of the culture injected and the survival time in doses up to $\frac{1}{16}$ of a 48 hour culture.

Animals given smaller doses (*i.e.* $\frac{1}{32}$ and $\frac{1}{64}$ of a culture) survived somewhat longer. All strains of the organisms used with one exception were equally fatal. Animals injected with the less virulent strain survived from 14 to 21 days compared to a survival time of 3 to 13 days for animals injected with the other strains. Because of the lack of strict correlation between dose or strain and survival time, these factors have not been considered in the summary of data presented in Tables I and II. Probably because of the short incubation time in inoculated animals,

TABLE I

The Blood Picture of Carp Inoculated with B. salmonicida

Series 1

Animal No.	Days after inoculation	WBC	Sugar	NPN	Amino acid N	Urea	Uric acid	Creatine and Creatinine	Creatinine
		thou. per cu. mm.	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
20A	—	3.714	97.5	32.7	18.4	4.0	2.81	3.6	.70
20B	—	3.458	100.0	34.2	20.0	2.6	3.18	5.0	.87
4357	3		5.8	35.7	21.6		5.47	40.0	
4361	3		6.4	24.6	22.4		5.00	27.8	2.48
4362	3		7.3	24.0	21.6		5.00		
4374	3		12.3	37.0	27.4			38.6	
4359	4	4.400	45.5	174.2	36.2			2.4	.79
4360	4	4.380	57.5	422.4	99.5			17.0	
4366	4	4.350	63.8	221.9	193.5			16.0	
4372	4		33.1	234.1	126.4		8.74	35.8	
4359	11	3.740	37.7	299.3	131.5	96.2	2.32	4.1	
4364	11	13.380	70.5	227.0	112.0	52.9	2.84	20.9	
4366	11	5.600	82.5	261.5	177.9		2.88	13.6	1.80

the gross characteristic lesions of "Furunculosis" were infrequently observed, and then only in the individuals which survived 12 days or longer.

Three days after inoculation (Table I), analysis of the blood filtrate from four animals which were lying on their sides, near death, indicated that the blood sugar had fallen from the normal of approximately 100 mg. per cent (9) to the abnormally low level of 5.8 to 12.3 mg. per cent, while creatine (together with creatinine) had increased to 27.8 to 40.0 mg. per cent as compared to approximately 3 mg. per cent in normal carp (Table I). The latter value, however, was not accepted without

reservation since this increase in "creatine" did not visibly affect the total non-protein nitrogen content of these samples. Behre and Benedict (11, 12) have presented strong evidence that chromogens other than creatinine are capable of producing a color with Jaffe's reagent. Thus some unknown chromogen resulting from the treatment of the blood filtrate with hydrochloric acid may have provided the increased amounts of "creatine." Increases in "creatine" were often encountered in other inoculated carp.

TABLE II

The Blood Picture of Carp Inoculated with B. salmonicida
Series 2

Animal No.	Days after inoculation	WBC	Sugar	NPN	Amino acid N	Urea	Uric acid	Creatine and Creatinine	Creatinine
		thou. per cu. mm.	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
22A	—	4.150	73.4	34.2	17.0	3.1	3.18	6.2	.87
24A	—	1.753	71.0	36.2	21.0	2.0	3.11	6.4	.90
24B	—	3.958	67.6	31.7	16.8	1.8			.70
4352	4		22.6	691.0	409.5	101.0		22.0	
5T	4		32.5	791.0	500.0	159.0		46.0	2.15
15T	4		52.5	120.3		14.6		23.5	
21T	4		11.6	173.3	103.0	19.9	5.02		
4351	7	5.480	17.0	296.0	206.0		3.41	7.6	.72
13P	7	2.470*	106.0	393.5	244.0		4.15	9.6	
23T	7	1.368*	20.6	290.0	264.0		5.43	28.4	1.90
13P	12	3.480*							
15P	12	6.830	115.7	206.1	132.0	31.9		3.8	1.10

* A small number of irregular giant white cells (leukoblasts) with multi-lobed nuclei were observed in these fish.

On the fourth day (Table II) concurrent with levels of blood sugar somewhat increased over the previous day but which were still subnormal, the non-protein nitrogen content of the samples had increased to phenomenal amounts. Values of 600 to 700 mg. per cent were not uncommon, while analysis of control samples remained at approximately 30 mg. per cent. For the most part, the increased nitrogen consisted of amino acid nitrogen. This constituent had increased to 400 to 500 mg. per cent in some animals. Variable increased quantities of urea were present which, in some cases, accounted for a substantial fraction

of the rise in nitrogen in the samples. Variations in other constituents were not consistent when analyses were performed at various intervals until 12 days after inoculation.

The hemoglobin, red blood cell count, and the total plasma protein, albumin, and globulin in the inoculated fish were essentially the same as in control animals. Although the fibrinogen content appeared to be normal in the blood of the diseased fish, a marked qualitative change in this constituent appeared. When calcium chloride solution was added to oxalated plasma which had been diluted with saline solution (13), a rapidly flocculating, granular, non-gelatinous precipitate formed instead of the firm cohesive clot obtained from the plasma of normal fish. An unusual amount of oil globules was always observed on the surface of accumulated visceral fluid of autopsied animals. Likewise, oil globules, never observed in control samples, were present in blood taken from all diseased carp.

It has been previously observed in studies on the nature of the lesion in the disease, that no cellular reaction or leukocytic infiltration into the inflammatory foci could be seen (4). Similarly, no general leukocytosis was indicated in a large series of white blood cell counts. In only one animal was the total count increased somewhat (Table I); in 4 others (Table II) a small number of leukoblasts (irregular giant whites) were observed.

The 10 carp injected with the less virulent strain in *B. salmonicida* did not exhibit variations from the normal until 7 days after injection. In only a few of these animals was the initial reduction of blood sugar observed; the characteristic rise in non-protein nitrogen also appeared to progress less rapidly. Since the average survival time of these fish (18 days) far exceeded that of animals injected with the other strains (6 days), it would appear that the severity of the disease was not manifest until shortly before death. One animal, 15P (Table II), which was inoculated with this strain presented a large external ulcer at 12 days, and correspondingly a typical increase in non-protein nitrogen was found. This animal died 14 days after inoculation.

Discussion

While the present data are insufficient to outline completely the progress of the organism *Bacterium salmonicida* in diseased fish, these experiments nevertheless suggest that the first pathological reaction in an acute infection is a rapid fall in blood sugar. This response appears

to be so drastic that the possibility of a hypoglycemic shock may be considered to offer a partial explanation for the rapidity of death (within 3–4 days) after inoculation, since other changes were not obvious. In a more chronic infection, outstanding, as might be predicted of a proteolytic organism, were the changes undergone by the nitrogenous constituents of the blood. The very high concentrations of total non-protein nitrogen (which reached levels of almost 800 mg. per cent) are probably as high as any hitherto recorded under experimental conditions in any animal.

In summary, our data suggest the following reactions to *Bacterium salmonicida* in the carp; when the inoculum is large, the rapidly multiplying organism utilizes the blood sugar as a convenient source of energy, inducing a hypoglycemic shock which may be fatal. The non-fatal induction period permits the slower process of invasion and proteolysis of muscle tissue (4, 5). The latter process releases breakdown and excretory nitrogen products which accumulate in the blood stream concurrent with or as a result of the kidney degeneration that is characteristic of infection with *B. salmonicida*. Thus the increased quantities of urea may only reflect a progressive uremia. The increase in amino acids as well as in creatine, is probably a result of an intensive degeneration of muscle tissue. It is conceivable that creatine can be utilized by the bacteria as a ready source of energy (14).

Conclusions

1. Furunculosis was induced in carp by the artifical inoculation of the fish with *Bacterium salmonicida,* and a detailed study was made of the blood of the injected animals.

2. Profound alterations occurred in the blood of the diseased fish. The concentration of blood sugar fell initially to extremely low levels and then recovered somewhat. Marked changes were observed in the non-protein nitrogen fraction of the blood. The concentration of amino acids underwent a many-fold increase (up to 500 mg. per cent), while lesser increases in urea and in creatine were noted.

3. No consistent quantitative changes were observed in the numbers of red and white blood cells, in hemoglobin, or in total plasma protein. However, the fibrin clot from the plasma of diseased fish was granular and flocculent in contrast to the firm cohesive mass formed from normal plasma.

4. These gross changes in the composition of the blood appeared to be

related to the proteolytic power of the organism and its known capacity to cause the disintegration of muscle, kidney, and other tissues.

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Purification, Sedimentation, and Serological Reactions of the Murine Strain of SK Poliomyelitis Virus*

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Introduction

The adaptation, by Jungeblut and collaborators, of the SK strain of human poliomyelitis virus from monkeys to cotton rats, mice, and guinea pigs, and the symptomatology, pathology, and serology of the disease in these rodents have been studied in several papers (1, 2, 3). The present article describes a method for the extraction of this virus from infected mouse brains and offers some data on the sedimentation, electrophoretic mobility, and serological reactivity of purified virus preparations.

The material used in this investigation was derived from the 270th serial mouse passage of the virus, and from subsequent passages up to the 320th, approximately. Throughout the study period of about one year the characteristic features of the strain, in particular its high infectivity, remained unchanged. All titers given were obtained by the intracerebral inoculation in young Swiss albino mice of 0.03 cc. of serial tenfold virus dilutions; four to six mice were inoculated with each dilution, and the titers were estimated statistically. As a rule, a fresh 10 per cent suspension of infected brains contained from 10^7 to $10^{7.5}$ M.L.D. per inoculum.

I. Purification of the Virus

Method

The method previously described (4) was modified as follows: Albino mice were inoculated either intracerebrally (0.03 cc.) or intraperitoneally (0.1 cc.)

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with a 10^{-5} suspension of infected mouse brains. The animals were killed when they showed peripheral paralysis or marked encephalitic symptoms, and the brains were removed and occasionally stored for a few days in the refrigerator. Freezing of the brains, however, contrary to what had been stated earlier (4), was found inadvisable. The brains were ground by hand in a mortar to an homogeneous paste and nine volumes of saline, containing 0.1 per cent glycine, were slowly added with mixing; glycine was used systematically with all our preparations because of its pronounced protective effect of virus in dilute solution.[1] The 10 per cent brain suspension was centrifuged for ½ hour at top speed (4800 r.p.m.) in 80 cc. celluloid tubes of the Swedish angle centrifuge. The supernatant thus obtained was fairly clear and could be easily decanted. Its total protein content (as estimated by Kjeldahl after dialysis) was about 0.2 per cent. We shall subsequently refer to this solution as Preparation I, or Solution I.

A volume of ether equal to about $\frac{1}{10}$ that of Preparation I was placed in a flask; the virus solution was then poured into it and the two fluids were mixed by transferring them gently from one flask into another a few times. Most of the ether dissolved into the virus solution, which was then poured into a graduated cylinder; excess ether collected at the top and was pipetted off. A solution consisting of half M acetic acid and half M sodium acetate, equal in volume to $\frac{1}{4}$ the virus solution, was now slowly added to the latter with constant stirring, thus lowering its pH to 4.6; a precipitate immediately formed which was easily centrifuged down at low speed. The supernatant was discarded, and the centrifuge tubes were inverted and allowed to drain. A few stainless steel balls were then placed in the centrifuge tubes and these were gently shaken while a solution of 0.12 M Na_2HPO_4, containing 0.1 per cent glycine, was added drop by drop to the precipitate; a homogeneous resuspension of the precipitate was thus achieved. The total volume of phosphate solution added was equal to $\frac{1}{10}$ that of the ether treated solution; and the pH was thus brought back to about 7.2. The suspension was now placed in the flat (4 mm. in thickness) tubes of the Swedish angle centrifuge, and spun for ½ to 1 hour at 4800 r.p.m. The supernatant thus obtained was almost water clear, or slightly opalescent; its protein content was of the order of 0.05 per cent. We shall refer to it as Solution II, or Preparation II.

Solution II was placed in the concentration ultracentrifuge and spun for an average of 40 minutes at 36,000 r.p.m.; the tubes were then inverted and allowed to drain and the sediment resuspended in a small volume of phosphate buffer or saline (about $\frac{1}{40}$ the volume of Preparation II). The suspension was spun for ½ to 1 hour in a flat tube in the Swedish angle centrifuge and yielded a slightly opalescent supernatant, the protein content of which was of the order of 0.01 per cent. We refer to this as Solution III, or Preparation III.

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[1] The infectivity of a thousandfold dilution in saline of the original brain suspension was found to disappear almost completely after a few days' storage in the ice-box, but persisted nearly unchanged when a little glycine (0.1%) was added to the solution. The protective effect of glycine on the diphosphopyridine protein of yeast has been ascribed to the fact that glycine binds metal ions responsible for denaturation: E. Negelein and H. J. Wulff, *Biochem. Z.* **293**, 351 (1937).

Results

The results from eight virus purifications, carried out by the above technique, are summarized in Table I. The protein concentrations given were estimated by Kjeldahl analysis for Solution I, and by sulfosalicylic acid precipitation and comparison with known standards for Solutions

TABLE I

Potency of Purified SK Murine Virus Preparations

Experiment No.	Preparation No.		
	I Original suspension	II Soluble fraction of acid precipitate from I	III Soluble fraction of ultracentrifuge sediment from II
	Volume		
	400 cc.	40 cc.	1 cc.
	Approximate Protein Concentration		
	0.2%	0.05%	0.01%
	Titer (log of M.L.D. per inoculum)		
1*	7.0	8.0	
2†	7.5	8.5	
3†	ca. 7.5	ca. 8.5	
4‡	7.2	8.7	
5‡	<6.5	8.2	9.5
6‡	7.5	>8.5	
7‡	7.2	7.8	
8‡	7.3	8.8	9.7

* Brains kept frozen 6 weeks.
† Brains kept at +1°C. 4 weeks.
‡ Fresh brains.

II and III. The degree of purification reached is indicated by marked increase in virus titer with simultaneous decrease in total protein concentration.

At icebox temperature, Preparation III remained stable for at least a few days, though its titer appeared to drop fairly rapidly. The Molisch test indicated the presence of but little carbohydrate. When an older preparation, which had been thoroughly dialyzed against distilled water, was examined between crossed polarized sheets, some precipitated ma-

terial which it contained showed double refraction, while the rest of the solution showed double refraction only upon flowing.[2] This suggested the presence of elongated molecules, some of which may have formed into aggregates approaching the crystalline state.

The method of purification just described was evolved by purely empirical means and after much random experimentation. It should be mentioned that the principle on which it rests—acid precipitation of the brain proteins followed by elution of the precipitate in neutral buffer—was simultaneously applied by Racker (5), with a degree of success which cannot be assessed for lack of adequate data. Since the literature offers but little information on normal brain proteins (reviewed by Block and Brand, 6), the present results throw no light on the relation of the virus to normal brain components; they show, however, that virus in infected brain is but an infinitesimal part of the total brain proteins. Assuming that all the material in Preparation III was virus, 1 cc. of the latter contained 0.1 mg. of virus. Since this was derived from 400 cc. of 10 per cent brain suspension, itself coming from about 40 g. of brain with a content of 4 g. brain protein, it will be seen that the virus thus extracted represented $\frac{1}{40,000}$ of the total proteins of brain. In reality the virus in Preparation III was probably still far from pure so that, even allowing for some loss in the course of the extraction, the actual ratio was probably much nearer one part in 100,000.

II. Sedimentation of Purified Virus[3]

In order to obtain sufficient material to yield a visible boundary in the ultracentrifuge, it became necessary to concentrate the virus preparations to an extent far greater than afforded by the method described above. Seven hundred infected mouse brains were collected, from which 260 cc. of Preparation II was obtained. This fluid was subjected to ultracentrifugation in five successive lots, the sediments being allowed to remain in the tubes while the supernatants were poured off and replaced with fresh solution. The sediments were then resuspended in a

[2] We are indebted for these determinations to Dr. D. Harker of the General Electric Company, Schenectady, N. Y.

[3] Work on the sedimentation and electrophoresis of the virus was begun in collaboration with Dr. D. H. Moore of the Department of Anatomy, College of Physicians and Surgeons, Columbia University, but was unfortunately interrupted before it could be completed. We are indebted to Dr. Moore for permission to use the data reported here.

total volume of 0.4 cc. of phosphate buffer, pH 7.2. The suspension thus obtained was only partially clarified after one hour's centrifugation in the Swedish angle centrifuge and remained appreciably milky. The concentration thus achieved was about sixteen times that obtained by the method described in the preceding section; the protein content of the solution may therefore be estimated at 0.2 per cent. When analyzed in the sector cell of the ultracentrifuge, this solution showed two boundaries, one with a slow sedimentation rate, and the other with a sedimentation rate of about 60 S. After threefold dilution in buffer, the distinction between the two boundaries became clearer, the faster one now moving at the rate of about 80 S. The two components appeared to be present in about equal concentrations.

The original specimen was then diluted 25 times in saline, spun again in the concentration ultracentrifuge, and the sediment taken up in a volume of saline equal to double the original amount of fluid. This procedure removed the slowly sedimenting component from the solution, leaving only one boundary which had a sedimentation rate of about 130 S and showed comparatively little spreading.

In view of the very high infectivity: protein ratio found with preparations obtained by this procedure, and because the bulk of virus infectivity was known to be associated with rapidly sedimenting material, the evidence is consistent with the assumption that the fast boundary observed was that of the virus itself. These results crude though they are, suggest that the rate of sedimentation depended greatly on buffer composition and on virus concentration. Inasmuch as marked increase in sedimentation rate with dilution is characteristic of solutions containing elongated molecules, such as tobacco mosaic virus (7) or linear organic polymers, this observation is consistent with the assumption that poliomyelitis virus activity may be predominantly associated with particles of thread or rod-like shape.

In their publication on mouse encephalomyelitis virus, Gard and Pedersen (8) reported that samples of purified virus contained three fast sedimenting fractions, with rates of 40, 160, and 210 S, respectively; they concluded that the mid fraction represented the virus because it failed to appear in normal mouse brain similarly treated. Our figure of 130 S for murine SK poliomyelitis virus would therefore be in approximate agreement with their's, as well as with a sedimentation rate of the order of 200 S previously estimated indirectly by sampling methods (4). Gard and Pedersen's results, however, were not described in detail, and no

mention was made of the possible effect of concentration on sedimentation rate.

When normal brain, in smaller amounts, was subjected to the same procedure used to purify infected brain, a final Preparation III was obtained with about the same protein content as virus Preparation II. A comparative study of these two fractions under the electronmicroscope, which has been reported elsewhere (9), suggested that there were physical differences between the virus and the normal preparation; material reasons, however, prevented a more extensive study of the latter from being carried out.

III. Electrophoresis of the Virus

Since lack of sufficient material prevented studying the electrophoresis of purified virus preparations by direct optical observation in the Tiselius apparatus, the indirect method, applied to unpurified brain suspension, was resorted to. It consisted in withdrawing a series of samples from the ascending side of the small electrophoresis cell after the completion of a run, and testing the samples for virus activity.

TABLE II

Electrophoresis of Unpurified SK Murine Poliomyelitis Virus

Buffer	*pH*	*Ionic Strength*	*Mobility* $\times 10^5$
NaCl-Phosphate	7.1	0.2	2.0
NaCl-Borate	8.4	0.1	5.3

Two experiments were made. In one case a 30 per cent normal mouse brain suspension, clarified by centrifugation at 4800 r.p.m. for one hour, was dialyzed against buffer 0.15 *M* in NaCl, 0.02 *M* in PO_4, pH 7.1, ionic strength 0.2, and a small amount of highly diluted infected brain suspension in the same buffer was then added to it. In the other case a 30 per cent infected brain suspension was centrifuged and dialyzed against a NaCl-borate mixture, pH 8.4, ionic strength 0.1. The serial samples, which measured 6 mm. in height, were withdrawn with a capillary pipette and titered for infectivity. In spite of the absence of visible boundary disturbance during sampling, some inaccuracy was introduced by the presence, owing to the narrowness of the cell, of a pronounced meniscus. The position of the boundary was estimated from that of the first sample (counting from bottom up) which showed a tenfold drop in virus titer. The titrations did not suggest an undue

degree of boundary spreading; since, however, many more determinations would be necessary for an accurate measurement of electrophoretic mobility by this method, the results reported should be considered as approximations only.

Table II indicates that the pH mobility curve of the virus was fairly steep, and that its iso-electric point was about pH 6. Schlieren diagrams taken in the course of electrophoresis revealed the presence of several components in the brain solution; no definite correlation could be established, however, between any visible fraction and virus mobility. The few data on the electrophoresis of viruses thus far reported (10) suggest that viruses have pH-mobility curves of the same type as most proteins.

IV. Precipitin Reactions with Purified Virus Preparations

Attempts were made to examine the various purified preparations for their ability to yield specific precipitin reactions with convalescent and hyperimmune sera.

Antigens

They included (1) the original virus brain suspension, already referred to as Preparation I, which was clarified by shaking with ether and centrifuging at 4800 r.p.m. in the angle centrifuge; (2) the partially purified solution, called Preparation II; (3) the supernatant from the latter after ultracentrifugation; and (4) the resuspended ultracentrifuge sediment (Preparation III). Similar preparations from normal brain were tested simultaneously. Preparations II and III, obtained in phosphate buffer, were first dialyzed against saline; this caused the appearance of a slight precipitate which was easily removed by low speed centrifugation.

Antisera

The hyperimmune sera were prepared by injecting one rabbit with Preparation I, clarified by ether treatment, and another with a similar preparation from normal mouse brains. Beginning with 1 cc. intraperitoneally twice weekly, the dose was progressively increased to 5 cc. at the end of 5 weeks. Two weeks after the beginning, injections were also started intravenously, beginning with 0.5 cc. twice weekly and increasing to 2 cc. at the end of 3 weeks. The animals were bled one week after the last injection. Hyperimmune sera from two other rabbits were also used, which had been immunized in the same way (except that ether treatment was omitted) with cotton rat brain infected with either Theiler's mouse virus (11) or with SK murine virus.

The convalescent sera included sera from monkeys convalescing from infection with the Aycock or the SK strain of poliomyelitis virus; one convalescent serum from a guinea pig paralyzed by the cavian strain of SK poliomyelitis virus; three human convalescent sera from a poliomyelitis epidemic in Westchester County, N. Y., from which Jungeblut and Dalldorf (12) have recently isolated a new rodent poliomyelitis virus known as M.M. strain; and, finally, pooled sera from hamsters

convalescing from infection with rodent passage virus of the M.M. strain. A number of normal sera were used for controls.

Precipitin tests

A layer of the antigen solution to be tested was placed over a layer of serum in small test tubes measuring 2 mm. in diameter; the tubes were incubated for 30 minutes at 37°C. and allowed to stand another 30 minutes at room temperature before reading for rings. The contents were then mixed, incubated for 1 hour at 37°C. and read again after 48 hours in the icebox. The reactions were graded ++, +, ± and 0. In general, all ++ tubes gave, after mixing, definite flocculation, while + tubes often became doubtful after mixing, especially when the control serum itself was not sufficiently clear. Only the results from ring tests will therefore be reported here.

Results

Table III summarizes the results obtained when Preparations I, II, and III from SK virus mouse brains, as well as similar preparations from normal brains, were tested with hyperimmune sera against unpurified SK virus mouse brain or unpurified normal mouse brain. It will be seen that while marked positive reactions were observed, in all tests, with Preparations I and II (including Preparation II after ultracentrifugation), purified Preparation III gave definitely positive reactions only with virus brain antiserum.

In further experiments virus Preparation III, in three-fold serial dilutions, was tested against the same two hyperimmune sera (Table IV). Six different batches of Preparation III were used. Samples 1, 2, and 3 were prepared according to the method outlined in the beginning of this paper; Samples 5 and 6 were prepared by the same method, but from material which, presumably through too vigorous ether treatment, had lost at least 90 per cent of its virus infectivity; Sample 4 was a more concentrated preparation, which had been subjected to a second ultracentrifugation in order to remove low molecular weight material. It is obvious from Table IV that the antigen always gave an appreciably higher (or, in number 4, considerably higher) titer when tested against antivirus serum than when tested against antinormal serum; besides, there was a perceptible correlation between antigen titer and virus titer, as shown in the last column of Table IV. Additional evidence of the specificity of these reactions was furnished by the fact that after a mixture of virus Preparation III and antiviral hyperimmune serum had been allowed to flocculate, the supernatant failed to give a ring when tested anew against virus Preparation III, whereas a marked ring was obtained when tested against either virus or normal Preparation II.

TABLE III

Precipitin Reactions (Ring Test) with Preparations from SK Murine Virus Brain (V) or Normal Brain (N) and Hyperimmune Rabbit Sera

	Antigen			
	V	N	V	N
	Serum			
	Anti-virus Brain		*Anti-normal Brain*	
Original suspension (I)................	++	++	++	++
Partially purified prep. (II).............	++	++	++	++
Purified prep. (III).....................	++	0	0 or +	0

TABLE IV

Precipitin Reaction (Ring Test) with Preparations of Purified SK Mouse Virus and Hyperimmune Rabbit Sera

Sample Number	Serum	Antigen Dilutions				Saline	Infective titer (undiluted) *log M.L.D.*
		1	1:3	1:9	1:27		
1	V	++				0	9.5
	N	0				0	
2	V	++	+	0		0	9.7
	N	+	0	0			
3	V	++	++	+	0		9.3
	N	+	0	0	0		
4	V	++	++	++	±		
	N	±	0	0	0	.	
5	V	+	0	0			Ca. 8.0
	N	0	0	0			
6	V	0±				0	8.3
	N	0					

V = anti-SK virus mouse brain serum; N = anti-normal mouse brain serum.

In Table V data have been gathered concerning the reactions between virus Preparation III and several immune and normal sera. It will be seen that Aycock convalescent monkey serum, SK convalescent monkey

serum and SK convalescent guinea pig serum gave markedly positive tests; postive tests were also obtained with two out of three M.M. convalescent human sera, and with pooled M.M. convalescent serum from experimentally infected hamsters. The test were also positive with two

TABLE V

Precipitin Reactions (Ring Test), with Preparations of Purified SK Mouse Virus and Normal Sera, Convalescent Sera, or Hyperimmune Sera

Serum	Antigen		
	Virus Preparation III	Virus Preparation II	Saline
Monkey Aycock convalescent	++ ++		0
Monkey SK convalescent	++ ++ ++	0	±
Guinea pig SK cavian convalescent (pooled)	++		0
Hamster MM convalescent (pooled)	+		0
Human MM convalescent #1	± 0	0 0	
#2	+ +	± ±	
#3	+ +	0 0	
Rabbit, cotton rat Theiler hyper-immune	++		0
Rabbit, cotton rat SK hyper-immune	++		0
Normal monkey	0		0
Normal guinea pig #1	0	0	
#2	0 +	0	
#3	0	±	
Rabbit, antisheep hemolysin	0	0	0
Human #1	0 0	0	0
#2	+ +	±	0
#3	0 0	0	0

The number of symbols for each serum expresses the number of different antigen preparations against which the serum was tested.

hyperimmune rabbit sera against cotton rat virus strains (Theiler or SK); however, the possibility of cross reaction between cotton rat brain antiserum and mouse brain antigen (13) was not investigated, and the positive reactions obtained with these sera may not have been wholly specific. On the other hand, all eight normal sera, with the exception of

one, were negative, the exception being offered by a human serum the history of which could unfortunately not be traced.

Discussion

The results just reported may be taken to indicate an appreciable degree of purity of the infective material thus isolated. How much normal high molecular weight protein it still contained can unfortunately not be stated. The lowest protein concentration at which the purified virus preparation gave a positive ring test in the present experiments was, at most, 10^{-5} (see Table IV), while flocculation in concentrations up to 10^{-6} and 10^{-7} has been reported with purified plant viruses (14), which are also high molecular weight proteins. It may thus be that the material here isolated was still 90 to 99 per cent impure, though the conditions under which the tests were performed did not make for great sensitivity. On the other hand, assuming for the virus a molecular weight of 10 million (which corresponds approximately to a sedimentation constant of 130 S), the high infectivity/protein ratio of the purified preparations leads to the conclusion that something of the order of 100 molecules represented one infective dose.[4] Since at least one molecule should be necessary to initiate virus propagation, the purified virus preparation, on this basis, could not have been more than 99 per cent impure. Unfortunately, there is no evidence that molecules with a molecular weight of 10 million represent the ultimate active units of the virus. In fact, filtration (15, 16, 17) and diffusion (18) experiments carried out on several strains of poliomyelitis virus suggest that the actual units may be appreciably smaller. The degree of purity of the virus thus extracted therefore remains conjectural.

The reactions observed between purified virus preparations and immune sera are significant in that they suggest that sera of animals and human beings convalescing from infection with several strains of poliomyelitis virus are able to react specifically *in vitro* with concentrated

[4] Taking for instance Experiment No. 8, Table I: Preparation III contained 0.01 per cent protein, or 10^{-4} g. per ml. Since it was still active after being diluted $10^{9.7}$ times, it was still active when it contained $\frac{10^{-4}}{10^{9.7}} = 10^{-13.7}$ g. protein per ml. One inoculum measuring 0.03 ml., one inoculum contained $10^{-13.7} \times 0.03 = 10^{-15.2}$ g. protein. The weight of one molecule from a substance with a molecular weight of 10,000,000 being $\frac{10^{7}}{6 \times 10^{23}} = 10^{-16.8}$ g., the number of molecules per M.L.D. was therefore $10^{-15.2}:10^{-16.8} = 40$.

antigen from the SK mouse strain. The failures of previous investigators in this field may be primarily ascribed to the lack of sufficiently potent antigens. Since virus activity and precipitinogen were found in the same fast sedimenting fraction, it is improbable that any "soluble" antigen, distinct from the virus, was present in appreciable amounts.

Summary

1. A method has been described for the extraction of SK murine poliomyelitis virus from infected mouse brains, which permits an appreciable purification of the virus with little loss.

2. The sedimentation rate of the purified virus preparation ranged from 60 to 130 S and seemed to depend greatly on concentration. This suggested a molecular weight for the virus of the order of 10 million.

3. The approximate electrophoretic mobility of the unpurified virus was 2.0×10^{-5} at pH 7.1, ionic strength 0.2, and 5.3×10^{-5} at pH 8.4, ionic strength 0.1.

4. The purified virus preparation yielded positive precipitin reactions with rabbit serum prepared against infected mouse brain, and doubtful or negative reactions with rabbit serum against normal mouse brain. Positive reactions were also obtained with sera from humans and animals convalescing from infection with various strains of poliomyelitis virus, while control sera, in general, were negative.

After completion of this work there became available the report of a similar investigation carried out by Gard in collaboration with Svedberg and Tiselius in Uppsala, Sweden: Sven Gard, Purification of poliomyelitis viruses, *Acta Medica Scandinavica*, Supplementum, **143** (1943). The data given for purified preparations of Theiler's spontaneous mouse encephalomyelitis virus ("mouse poliomyelitis") and of human poliomyelitis virus are in substantial harmony with those reported here for the mouse-adapted strain of human SK poliomyelitis virus. Thus, the sedimentation constant for purified mouse virus was found by the Swedish workers to be of the order of $150 - 160 \times 10^{-13}$, that of purified human virus 150×10^{-13}. The apparently close agreement in the physical properties of the three strains of poliomyelitis virus examined is noteworthy.

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Heat Inactivation of the Murine Strain of SK Poliomyelitis Virus*

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This paper presents a brief study of the inactivation of the murine strain of SK poliomyelitis virus under the influence of heat. For information concerning the physical nature of the virus, the preparations used, and the method of titration, the reader is referred to the preceding paper (1).

Experimental

Various virus preparations were placed in test tubes and incubated in a thermostat the temperature of which remained constant within about 0.2°C. In the experiments at higher temperatures only 0.05 cc. of virus preparation was placed in small, thin test tubes, about 0.5 cc. in capacity; these were first immersed in ice water, then in the bath, then in ice water again, to insure uniformity of heating and cooling. The samples were then tested for virus activity by the usual procedure.

Results

Table I shows the effect of heating for 5 minutes at 56.5°C. the original 10 per cent brain suspension in saline and 0.1 per cent glycine, which had been clarified by low speed centrifugation. The significant fact was the appearance of completely erratic titrations, which were never observed with unheated material. Heating increased the opalescence of the solution, but did not cause it to precipitate.

When the partially purified virus preparation, referred to in the preceding paper as Preparation II, was used instead, much more regular

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titrations were obtained at 49.5°C. and 56.5°C., and titers could be estimated with fair accuracy. At 63°C., however, inactivation was so rapid that only a trace of infectivity was left after 1 or 2 minutes. The results are summarized in Fig. 1. At 49.5°C., the relationship between logarithm of infectivity and heating time is approximately linear, while at 56.5°C. the reaction rate decreases appreciably with time; the curve drawn for 63°C. is only a coarse approximation.

In order to determine how much, under similar experimental conditions, the inactivation rate of virus was dependent on virus concentration alone, the following experiment was carried out. The original virus suspension in saline containing 0.1 per cent glycine was centrifuged at 5000 r.p.m. until fairly clear, and then diluted in normal mouse brain suspension similarly prepared; the samples were then incubated at 56.5°C. for 6 minutes and titered. Titration results were fairly sharp, and virus titers could be estimated without difficulty. Table II shows that 6 minutes heating caused a drop in titer of about 3 log in all samples; inactivation was therefore, under such conditions, not grossly dependent on initial virus concentration.

TABLE I

Heat Inactivation of Virus in Original Suspension at 56.5°C.

Log of Virus Dilution	Time (minutes) 0	5	10	15	20	30
8	D-----					
7	DDDDD-	--				
6	DDDDDD	--	--			
5		DD	--	D-		
4		DD	-----	DD		
3		DD	D-----	--	DD-	
2		DD	DDDDD	DD	---	D--
1		DD	DDDDD	DD	DD-	D--
0				DD	DDD	DDD

D = dead mouse.
- = surviving mouse, 7 days after inoculation.

Discussion

The heat denaturation of proteins and the inactivation of enzymes have usually been reported to be unimolecular rections (for extensive references, see Eyring and Stearn, 2), in which case plotting the logarithm of the remaining amount against time yields a straight line. A unimolecular reaction rate was also observed by Price (3) for the heat inactivation

of several plant viruses, while Lauffer and Price (4) observed that thermal inactivation of tobacco mosaic virus (measured by decrease in infectivity)

TABLE II

Heat Inactivation of Virus in Original Suspension Diluted with Normal Brain Suspension

Heating time: 6 minutes at 56.5°C.

	Log M.L.D.			
Titer before heating	6.7	5.7	5.5	3.5
Titer after heating	3.7	1.8	2.7	0.7
Difference	3.0	3.9	2.8	2.8

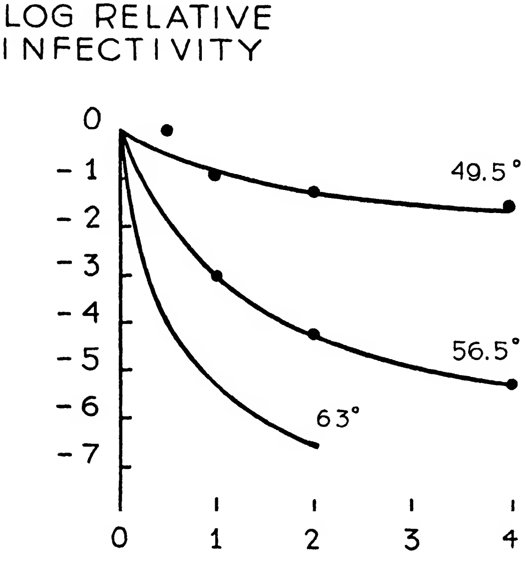

FIG. 1

Heat Inactivation of Partially Purified Virus in Phosphate Buffer, pH 7.2, Containing 0.1 Per cent Glycine

Time: in hours, for experiment at 49.5°C.
in minutes × 10, for experiment at 56.5°C.
in minutes, for experiment at 63°C.

The approximate composition of the buffer was as follows (see Ref. 1): *M* Na-acetate, 1 part; *M* acetic acid, 1 part; 0.15 *M* NaCl, 8 parts; 0.12 *M* Na_2HPO_4, 30 parts; glycine, 0.095 per cent.

proceeded at a considerably faster rate than thermal denaturation (measured by decrease in solubility).

In several instances, however, as in the case of invertase (5), amylase

(6), asclepain (7), antisheep hemolysin (8), diphtheria antitoxin (9), the reaction rate has been found to decrease with time. As regards bacteriophage, Nanavutty (10) found a decrease in the rate of heat inactivation with time, while Krueger (11), obtaining a strictly unimolecular reaction rate, ascribed Nanavutty's results to faulty titration. The reason for such discrepancies is actually not clear.

The results reported here suggest that mouse poliomyelitis virus inactivation at 56.5°C. deviated appreciably from a first order reaction, while at 49.5°C. the reaction may have been more nearly unimolecular; the data, however, are not sufficient to warrant a definite conclusion.

Exact formulation of reaction rates is not indispensable for the estimation of relative rates at different temperatures. This can be done by examination of graphical data. Indeed, Fig. 1 shows that there was, for instance, a drop of 1 log, or approximately 90 per cent of virus activity, in about 60 to 120 minutes at 49.5°C.; in about 2 to 5 minutes at 56.5°C.; and in probably less than 15 seconds at 63°C. This represents roughly a thirty-fold increase in reaction rate for each temperature increment of 7 degrees. The temperature coefficient was therefore $\theta^7 = 30$, $\theta = 1.6$. The energy of activation of the reaction may be estimated by application of Arrhenius' equation:

$$\log_e \frac{K_1}{K_2} = -\frac{A}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

(K_1 and K_2 being the velocity constants at absolute temperatures T_1 and T_2 respectively; A the energy of activation, R the gas constant.) Writing $K_1/K_2 = 30$, the value found for A, between 49.5°C. and 56.5°C., is about 100,000 calories. The temperature coefficient and energy of activation thus estimated are of the same high order of magnitude usually found for the denaturation of proteins (2).

Summary

The rate of thermal inactivation of unpurified and partially purified SK murine poliomyelitis virus has been determined at 49.5°C. and 56.5°C. The energy of activation of the reaction was of the order of 100,000 calories per mole.

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A Dietary Factor, Essential for Guinea Pigs [1]

II. A Comparative Study of the Creatine Excretion of Animals on a Diet Deficient in This Factor and in Vitamin E [2]

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Introduction

In the course of investigations by Wulzen and Bahrs (1, 2, 3) on nutritional requirements of planarian worms it was found that raw cream contained a dietary factor for guinea pigs differing from those previously described. The partial isolation of this factor from raw cream was described by van Wagtendonk and Wulzen (4). A characteristic feature of the deficiency of this factor[4] is a development of a stiffness in the wrists of the experimental animals followed by a stiffness in the elbows. Furthermore calcium depositions, often in large amounts, are found indiscriminantly throughout the whole body in the later stages of the deficiency.

Evans and Burr (5) were the first to observe that a paralysis of a part of the musculature of the body wall and of the posterior extremities occurs in young rats born from vitamin-E-deficient mothers. These observations were later confirmed by Goettsch and Pappenheimer (6), and by Olcott (7). As a consequence this paralysis was considered as one of the specific symptoms of vitamin E deficiency.

Early in the work on the muscular dystrophy caused by a lack of

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[2] From a thesis submitted by one of the authors (V.S.) as partial fulfillment of the requirements for the degree of Master of Science, Oregon State College.

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[4] We will refer to this dietary factor as the anti-stiffness factor.

vitamin E in the diet it was recognized by Goettsch and Brown (8) that the creatine content of the affected muscles was greatly lowered. This was confirmed by MacKenzie and McCollum (9) in their investigations of vitamin E avitaminosis in rabbits, and by Shimotori, Emerson, and Evans (10) for guinea pigs. Morgulis and Spencer (11) found that there existed a correlation between the muscular dystrophy and the urinary excretion of creatine. The latter is considerably increased in the advanced stages of the dystrophy. Morgulis (13) as cited by Verzár (14) followed the different stages of the muscular dystrophy in guinea pigs by the urine analysis for creatine.

In some respects, the symptoms caused by the absence of the anti-stiffness factor from the diet suggested those reported for vitamin E deficiency. The present investigation was undertaken in order to differentiate between the vitamin E avitaminosis and the deficiency of the anti-stiffness factor using as criterion the urinary creatine excretion.

Experimental

a) *Method*

Five groups of guinea pigs, a total of 28 animals, were used for this investigation. The first group of four animals was reared on a stock diet consisting of rolled oats, straw, and greens *ad libitum*. This group served as a control. The other groups received a diet which was free of vitamin E and deficient in the anti-stiffness factor. The diet was based upon that used by MacKenzie, *et al.* (15) and consisted of:

Casein (Smaco, vitamin free)	1050 g.
Dextrin (commercial cornstarch)	4830 g.
Yeast (Fleischmann's dried irradiated brewers' yeast)	700 g.
Salt mixture, as described by MacKenzie, *et al.* (15)	420 g.
Orange juice (separately each day)	1 cc./100 g. weight

The following supplements were prepared:

Supplement 1. A solution of the anti-stiffness factor in cottonseed oil (commercial Wesson oil). Twenty milligram of the crystalline[5] material was dissolved in 5 cc. ethyl laurate. This solution was diluted with 35 cc. of cottonseed oil.

Supplement 2. A solution of synthetic α-tocopherol (Merck) in cottonseed oil, containing 6 mg. per cubic centimeter.

[5] To be published.

These supplements were given orally to the different groups as indicated: Group II (6 animals): no supplement. Group III (6 animals): 0.25 cc. of Supplement 1 (125 microgram). Group IV (6 animals): 0.5 cc. of Supplement 2 (3 milligram). Group V (6 animals): 0.25 cc. of Supplement 1 and 0.5 cc. of Supplement 2. The animals were kept in metal cages, bedded on sawdust, and segregated as to group and sex. Each day one third of the animals were placed in metabolism cages. The next day a 24-hour urine sample was collected. In this way the urine of each guinea pig was analyzed for its creatine and creatinine content on every third day. No values for the food intake were collected. The procedure of Folin (16) was followed for the determination of the creatine and the creatinine. The wrist stiffness was determined frequently with the method described in the previous publication (4). The results are recorded in terms of a series of arbitrary figures. A normal joint is designated as *4*, a completely rigid joint as *1*. Intermediate conditions are indicated by such sumbols as *1.5*, *2*, *3*. The superscript E, as in 2E, indicates that the elbow as well as the wrist is stiffened. The superscript *P*, as in 4P, indicates that, although normal mobility has been regained, the joint is still painful under manipulation.

The animals were weighed before they were placed in the metabolism cages, and the weights were recorded.

b) *Results*

From Table I it is evident that the dystrophy, as indicated by the creatine/creatinine ratio developed very slowly. After seventy-nine days none of the experimental animals showed an appreciable creatine excretion. The animals looked healthy, gained regularly in weight, and did not show marked symptoms of the vitamin E deficiency. The animals which did not receive the anti-stiffness factor showed a constant increase in stiffness. It was therefore decided to accelerate the onset of the E deficiency by adding cod-liver oil to the diet. Although several investigators (6, 17, 18) have thought that cod-liver oil had a toxic effect, Madsen (19) and MacKenzie, *et al.* considered that the rôle of cod-liver oil was that of oxidizing vitamin E in the gut. Mattill and Golumbic (21) provided final evidence that no distinction need be made between a cod-liver-oil-induced muscular distrophy in rabbits and the nutritional dystrophy produced by lack of vitamin E. Cod-liver oil also accelerated the onset of the stiffness without having any toxic effects.

One cubic centimeter of cod-liver oil was given to each animal on

alternate days, while the regular supplements were given on the intervening days. It was found in other experiments that it is necessary to give these supplements in a double dosage in order to counteract the influence of the cod-liver oil. From the seventy-ninth day on the symptoms of the two deficiencies became more striking up to the end of the experiment.

The control animals in group one survived the experiment unimpaired. At no time during the experimental period signs of the two deficiencies developed. The weight was doubled at the end of the period.

TABLE I

Deficiency Indices

Day	Group I 4 Animals			Group II 6 Animals			Group III 6 Animals			Group IV 6 Animals			Group V 6 Animals		
	Weight	$\frac{Cr}{Crtinine}$	Stiffness	Weight	$\frac{Cr}{Crtinine}$	Stiffness	Weight	$\frac{Cr}{Crtinine}$	Stiffness	Weight	$\frac{Cr}{Crtinine}$	Stiffness	Weight	$\frac{Cr}{Crtinine}$	Stiffness
	g.			g.			g.			g.			g.		
1	419	0.61	4-4	393	0.31	4P-3	473	0.47	4-4	446	0.51	4P-3	435	0.49	4-4
53	564	0.38	4-4	474	0.94	3-2	518	0.98	4-4	494	0.39	3-2.5	512	0.27	4-4
79	628	0.17	4-4	463	0.63	3-1.5	488[a]	1.99	4-4	497	0.84	3-2	532	0.96	4-4
86	670	0.37	4-4	475	2.91	3-1	475	1.96	4-4	497	1.03	2-1.5	531	0.76	4-4
91	687	0.45	4-4	470	3.29	2-1E	479	0.81	4-4	482	1.09	1-1E	522	0.64	4-4
97	721	0.57	4-4	460	8.30[b]	1-1E	480	3.09	4-4	513	0.65	1-1E	560	0.69	4-4

The figures in this table are the average values for the groups.

Note: $\frac{\text{Cr}}{\text{Crtinine}}$ stands for the $\frac{\text{Creatine}}{\text{Creatinine}}$ ratio.

[a] One animal of this group had died from diarrhea on the seventy-sixth day.

[b] Two animals of this group died on the 98th day from severe deficiency.

The animals on the vitamin-E-free diet without any supplements developed a severe wrist stiffness and a severe creatinuria, indicating a muscular dystrophy. As the experiment progressed the animals lost weight, and their muscles, particularly in the hind-quarters, became weak, diminished in size, and seemed to lose coordination. When placed upon their backs they could regain their footing only with difficulty and in some cases not at all. When the experiment was terminated two of the animals had died, two were in an emaciated moribund condition, and the others of the group were stiff and becoming paralyzed.

The animals in group three developed a muscular dystrophy as evident from the creatinine excretion which, however, was not so severe as that in the animals receiving no supplement (Group 2). Due to the presence of the anti-stiffness factor given in Supplement 1 their limbs were limber and no stiffness had developed. Their weights were slow decreasing.

The animals in Group four which had been on the vitamin-E-free diet supplemented with α-tocopherol did not show any signs of muscular dystrophy, either overtly or through the creatinuria criterion. The stiffness was strongly developed. Their weights increased steadily.

The animals which had received both supplements while on the vitamin-E-free diet thrived well. They remained in good health throughout the experiment although they did not increase in weight as rapidly as the animals on the stock diet.

Summary

A deficiency of the anti-stiffness factor produces in guinea pigs a characteristic wrist-stiffness but has no significant effect upon the creatine excretion. Avitaminosis E produces a muscular dystrophy accompanied by creatinuria. On a combined deficiency these symptoms apparently develop simultaneously and independently of each other, resulting in a greater degree of paresis than that produced by a deficiency of either of the factors individually.

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The Content and Formation of Histamine in Fish Muscle

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Introduction

In the course of investigations on pharmacologically active substances in fish tissue, the presence of a compound which exerted biological activities similar to histamine was usually encountered in some of the fish extractions. Suzuki, *et al.* (1) seem to have been the first to describe the presence of histamine in tuna extracts, but further information as to content or manner of formation of histamine in marine fish has not been available. We, therefore, decided to investigate this question, hoping to gain some further knowledge on the origin and biochemical behavior of this physiologically important base.

Methods

Most of our experiments were performed with tissues from California sardines (*Sardina coerulea*), California mackerel (*Pneumatophorus jap. diego*), and albacore (*Germo alalunga*). These experiments were usually conducted immediately after the arrival of the fish at the cannery which was a matter of some 6 or 8 hours after the fish were caught. In some cases frozen bluefin (*Thunnus Thynnus*) and frozen bonito (*Sarda Chiliensis*) were also used.

The extracts were prepared in the following manner. The muscle was ground in a mortar with purified sea sand or comminuted in a Waring Blendor. Then 10 g. of the resulting paste were mixed thoroughly with 70 cc. of water and heated rapidly to 80°C. Hydrochloric acid was then added to pH 4.8, the volume brought to 100 ml. with water, and the mixture filtered. Prior to bioassaying, the solution was neutralized with solid Na_2CO_3 to pH 7.2.

The histamine content was determined on the isolated guinea pig intestine. In the following discussion we use the term "histamine" with the understanding that the biologically determined values include also the socalled "histamine-like substances" (Minard, 2; Guggenheim, 3). Preliminary experiments showed that the results obtained by this procedure were practically identical with those obtained by using trichloroacetic acid for purification of the extracts, (*cf.* Guggenheim, 3), and furthermore, that histamine added to the muscle could be satisfactorily recovered by this method.

For the isolation of histamine and the preparation of its salts, we used the

methods of Best (4), Koessler and Hanke (5), Eggerth (6), and Gale (7) with some modifications.

Experimental Part

I

We found that 0.1 ml. of a mackerel extract prepared by the described method when added to 25 ml. of a Ringer-Locke bath induced in many cases a very strong contraction of the guinea pig intestine. This action was comparable to the contraction produced by 0.1 γ histamine in 25 cc. solution.

The active substance contained in mackerel, sardine, and tuna extracts was identified as histamine by the following biological and chemical evidence.

1. 10 ml. of the fish extract were ashed and the residue dissolved in 10 ml. 5% aqueous HCl. After neutralization with Na_2CO_3 the solution proved to be nearly inactive, indicating that the contraction of the intestine was not due to cations (Mg or K) present in the extract.
2. The activity of the extract could not be inhibited by atropine treatment of the intestine. This excludes the action of a parasympathicomimetic substance.
3. Addition of arginine to the Ringer Locke solution ($M/2$) inhibited in a reversible manner the contraction produced by the fish extract. Such an antagonism seems to be characteristic for the histamine action (Edlbacher, 8).
4. The fish extract was concentrated ten times in vacuo and 0.1 ml. of this concentrate was injected subcutaneously into two guinea pigs. After two minutes dyspnoe was observed, and within seven minutes death occurred by asphyxia. The heart was still beating, and the lungs were distended as characteristic for the histamine action.
5. 5 ml. of the same concentrate injected intravenously into a rabbit in ether narcosis slightly increased the blood pressure to an extent comparable to that produced by a histamine solution of similar activity. The same amount of concentrate injected intravenously into a cat in Nembutal anaesthesia had a strong hypotensive action, as characteristic of histamine.
6. One ml. of the concentrated solution rubbed on the surface of the skin caused a very strong urticaria like local reaction. This similarity between the local reaction of histamine and of fish extracts was already observed by T. Lewis (9) who believed, however, that the active substance in fish extract is not identical with histamine.
7. The fish extract gave a very strong Pauly and Knoop reaction which was, however, stronger than could be accounted for by the biological activity of histamine alone. The presence of other imidazole derivatives may be responsible for this difference.
8. Press juice from sardines was concentrated 17.5 times in vacuo. A biological assay of this solution indicated the presence of 9.6 g. histamine in 1000 ml. from

which 4.5 g. were isolated in form of pure crystalline histamine-diphosphate,[1] *i.e.* a yield of 47% was obtained. As yet we have not been able to decide if the missing 53% was also histamine, or another substance with histamine like biological activity. Experiments still in progress seem to indicate that at least one part of this substance has properties slightly different from histamine.

9. The following derivatives were prepared from the phosphate: histamine picrate, m.p. 232° (cor.), histamine dihydrochloride, m.p. 240° (cor.), histamine base, m.p. 81–83° (cor.). These values and the melting point of histamine diphosphate are practically the same as quoted in the literature (Guggenheim, 3).

By the above experiments we believe to have proved that normal fish tissue contains histamine.

II

From our preliminary bioassays, we had already noted that the histamine content of different samples of the same species of fish varied so much that it could hardly be explained by the assumption of individual physiological variations. The limits of these variations were found to range:

in sardines: from 1.8 to 36.8 mg. per 100 g. tissue
in mackerel: from 0.9 to 23 mg. per 100 g. tissue
in other fishes: from 1.4 to 25 mg. per 100 g. tissue

Since the autolytic decomposition in fish starts immediately after being caught, we attributed the great variation in the histamine content to different degrees of autolysis. This assumption was supported by the papers of Silva (10) and Dragstedt (11) who claim that histamine is present in the tissues in form of inactive peptide from which it is liberated by enzyme action. Therefore, we determined first, the histamine content of mackerel which had been caught alive and frozen immediately in dry ice to assure maximum freshness. Its histamine content was the lowest observed, viz. 0.09 mg. per 100 g. of tissue.

In another experiment, we estimated the histamine content of mackerel caught 4 to 5 hours before assay and found it to be 2.3 mg. per 100 g. The fish was then incubated at 15°C., and the histamine content determined after 16, 24, and 48 hours. In order to prevent any artificial contamination the samples were taken from different parts of the body with sterile surgical tools. The incubated fish at the end of the experiment had no objectionable odor. The histamine values were after 16

[1] The weight of the actually isolated histamine diphosphate was 12.42 g. Histamine diphosphate contains 36.17% histamine.

hours, 23 mg.; after 24 hours, 48 mg.; and after 48 hours, 94 mg. per 100 g. of tissue.

In another experiment the histamine content of the fresh fish (4.5 mg.) increased to 150 mg. per 100 g. upon standing 24 hours at room temperature (24°C.).

These experiments proved that the extractable histamine content of the fresh fish is very low and that a postmortal formation or liberation of histamine takes place in the muscle.

In order to gain more detailed information on the nature of this process, we suspended 100 g. of mackerel muscle paste in 100 ml. of sterile distilled water, added 3% chloroform and 5% toluene, and incubated the suspension at 37°C. for 24 hours (Sample I). As a parallel, a piece of mackerel muscle was not ground but was kept without addition of preservatives for the same time in the incubator (Sample II). The histamine content after incubation (Sample I) was only slightly higher (0.3 mg. per 100 g.) than the initial value (0.1 mg. per 100 g.) but the histamine increased considerably in Sample II up to 24 mg. per 100 g.

We estimated also the free amino acid content of Sample I by Sorensen's formol titration and found an increase from 0.9 ml. to 2.8 ml. $N/10$ NaOH during the incubation, indicating the action of proteolytic ferments.

The above experiments show that the presence of antiseptic substances in Sample I inhibited the histamine formation, and that proteolytic ferments do not considerably increase the histamine content. We assumed, therefore, that in the other samples bacterial action was responsible for the formation of histamine. This assumption was based mainly on the experiments of Ackermann (12), Koessler, Hanke (5) and Gale (7), who demonstrated the bacterial decarboxylation of histidine to histamine.

That fish muscle samples always contain bacteria was shown by inoculating sterile bouillon with cautiously taken samples.

The topical distribution of histamine in the incubated fish also supports the assumption that it is of bacterial origin. We found repeatedly that the histamine content increased much faster in muscle samples taken from nearest the skin than in samples from a deeper location, indicating that a germ infiltration may be responsible for the histamine production.

Further evidence for the bacterial origin of the newly formed histamine is given by the following experiment: One mackerel was divided into two

halves. One part (I) was incubated for 16 hours at room temperature while the other (II) was first frozen for six hours then thawed and incubated for 16 hours. Since previous freezing should enhance autolysis, we expected a higher histamine content in the previously frozen material. However, the histamine content of Part I after incubation was 94 mg. per 100 g. and that of II only 21 mg. This unexpected result indicates that cold sensitive bacteria (Kiser and Beckwith, 13) which were killed in Part II were responsible for the main part of the histamine production in Part I.

Experiments are in progress to determine which bacteria are responsible for this rapid histamine formation in fish.

The foregoing results suggest that the post mortal increase in histamine in the incubated whole fish is not so much the result of liberation of preformed histamine groups from a protein as a new production from such precursors as histidine.[2]

Considering the recent publications of Trethewie (14), Silva (10) and Dragstedt (11) it seemed important to establish more conclusively the extent to which the liberation of already preformed histamine groups is involved in the histamine formation we observed. It seemed possible that we did not determine all the preformed histamine by our method of hot acid extraction of the tissue paste, and that it was the histolytic ferment of the contaminating bacteria which liberated, through destruction of cell elements, the already preformed histamine.

We, therefore, determined the total histamine content of the fish tissue by acid hydrolysis. Usually 20 g. of muscle paste was digested in 80 ml. of 7 N H_2SO_4 at 10 lb. pressure for 10 hours. The cooled, neutralized and filtered solution was used for assay of the histamine. Preliminary experiments showed that added histamine could be satisfactorily determined by this method. The histamine content of fresh frozen muscle estimated in this manner (0.12 mg. per 100 g.) was only slightly higher than found by the extraction method, (0.09 mg. per 100 g.).

We also determined by acid hydrolysis the total histamine content of fresh mackerel and of mackerel incubated for 16 hours at room temperature. The histamine content was 3.6 mg. before and 49 mg. per 100 g. tissue after incubation, showing that a substantial amount of histamine was produced from inactive precursors during the incubation.

These experiments support the claim that the post mortal increase in

[2] A method for determining spoilage in fish and canned fish products based on the increase in histamine content will appear elsewhere [*Food Research*].

histamine is not the consequence of liberation of preformed histamine units.

III

In order to determine the physical state in which histamine exists in normal protein, whether it be in an inactive peptide linkage as suggested by Silva (10) or in free form but occluded and retained by cell debris until liberated by fermentative destruction as suggested by Trethewie (14), the following experiments were conducted.

We prepared a fairly pure protein from fresh frozen mackerel by isoelectric precipitation and consecutive extraction. In this manner approximately 80% of the N-containing material was recovered. The dry protein contained 14.2% N and 0.4% ash. It was shown in unpublished experiments that this protein had a high biological value, since rats kept on diets containing it as the exclusive N-source grew better than on diets containing the same ratio of casein.

10 g. of this protein were digested with 100 ml. of 8 N H_2SO_4 at 10 lb. pressure for 10 hours. No histamine could be detected in the protein prepared from fresh frozen fish, indicating that the small amount of histamine present in the fresh fish is soluble and not attached to protein. We also prepared a protein from fish which had been incubated for several hours. Its hydrolyzate contained in all cases, considerable amounts of histamine even after several reprecipitations.

We tried to free the latter protein from the histamine by the following methods:

1. 10 g. of protein was triturated in a mortar with 2 g. of Na_2CO_3 and extracted for 24 hours in a Soxhlet apparatus subsequently with methanol, ethanol, isopropanol, amylalcohol, and chloroform. Even after this effective extraction the acid hydrolyzate of the protein still contained 0.3–0.5 mg. of histamine per 100 g.

2. 10 g. of protein were suspended in the middle compartment of an electrodialysis apparatus and the content of the cathod and anod compartments was replaced every 2–3 hours with fresh distilled water. After 26 hours of electrodialysis we found the cathod compartment free from histamine. The acid hydrolyzate of the protein however, even after this treatment contained 0.65 mg. histamine per 100 g.

3. We found in control experiments that after the addition of histamine to fresh frozen mackerel muscle, the precipitated protein contained only traces of the base and was entirely freed of it by reprecipitation, the histamine staying in the supernatant liquid. Therefore, we suspended 10 g. protein from incubated fish in 100 ml. of N/10 aqueous NaOH, and after heating for a few minutes to 80°C. precipitated the protein by neutralization with HCl. The supernatant liquid became free from histamine upon the third reprecipitation, the protein however, still contained 0.8 mg. histamine (*cf.* Büttner, 15).

Since we were unable to liberate by physical means all the histamine from the protein of incubated fish, we assumed that at least one part of the base produced by bacterial contamination is chemically attached to proteins. This linkage has been shown to be easily split by acid hydrolysis. Finally, we investigated whether or not this histamine can be liberated from the protein by enzymes.

Two different proteins were used as substrates, mackerel protein A which after acid hydrolysis did not show any histamine content and B which contained after reprecipitation 1.1 mg. % histamine.

The fermentation was performed with Pancreatin USP (Armour) or with a proteolytic ferment extracted from fish intestines with a pH optimum of 8.5 to 9.0.

TABLE I

Liberation of Histamine by Proteolytic Ferments

Sample No.	Protein 10 g.	Ferment	Amino N per ml. according to Sorensen in ml. $N/10$ NaOH Before Incubation	After Incubation	Histamine *in γ per ml.*
1	A	Pancreatin	0	5.2	5
2	A	Fish ferment	0	4.7	4
3	B	Pancreatin	0	5.6	15
4	B	Fish ferment	0	4.8	18
5		Pancreatin			4
6		Fish ferment			6

10 g. protein were suspended in 100 ml. H_2O. After addition of 0.5 g. of ferment, 3% chloroform and 5% toluene, the pH was adjusted with $Ca(OH)_2$ to 8.7 and the sample incubated at 31°C. for 48 hours. After this period the histamine and the total free amino acid content was determined by Sorensen formol titration in each sample. As control we incubated the suspension of the ferment itself without addition of protein.

We see from the table that the control samples (5 and 6) themselves contained histamine, probably liberated from the ferment mixture by autolysis. Protein A, which after acid hydrolysis proved to be histamine free, developed upon incubation (Sample 1 and 2) only as much histamine as could be obtained from the ferment suspension (Sample 5 and 6) itself. Protein B, however, (Sample 3 and 4) in which histamine could be detected after acid hydrolysis liberated histamine also in this experiment. These values were slightly higher than after acid hydro-

lysis, probably because of the histamine content of the ferments (Sample 5 and 6). The increase in amino acid content (formol-titration) proves that considerable proteolysis took place during the incubation.

The experiments prove that tryptic fermentation does not produce histamine from proteins which do not contain it as a building stone and that only preformed histamine units can be liberated by proteolytic action. At least a small part of the histamine produced by bacterial action remains within a protein complex. This result could be explained by the assumption that histidine groups need not be in a free form in order to be transformed into histamine groups. It seems that the ferments of certain bacteria are able to split the peptide linkage involving the carboxyl-group of histidine; the consecutive decarboxylation does not attack the peptide linkage by which the histamine group isattached to the rest of the protein molecule.

Summary

1. It was proved by biological and chemical evidence that the muscle tissue of marine fish contains histamine.

2. Based on biological assays, we conclude that:

(a) The histamine content of the fresh fish is very low but increases rapidly *post mortem* as a result of bacterial contamination.

(b) The histamine in fresh fish protein is present in the free form. A small part of the histamine, produced *post mortem* remains in peptide linkage within protein molecules.

(c) Proteolysis liberates by destruction of cell elements and also by splitting of peptide linkages preformed histamine only. Proteolytic ferments do not produce free histamine from protein which does not contain histamine groups.

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The Influence of Pantothenic Acid upon the Susceptibility to Pneumonia[1]

(With a Note on the Mechanism of the Action of Sulfapyridine in Pneumococcic Pneumonia).

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Introduction

Recently it was demonstrated in this laboratory (1) that addition of sulfapyridine to the diets of albino rats inhibited the action of pantothenic acid. Animals receiving one per cent of this sulfa-drug in the diet developed symptoms which appeared to be chiefly those of pantothenic acid deficiency. This condition could be reversed by adding an excess of calcium pantothenate to the ration. It has been shown elsewhere (2) that this vitamin is necessary for the nutrition of Types I, II, V, and VIII pneumococci. It occurred to us that these facts, taken together, might serve as a basis of a hypothesis for the mechanism of the action of sulfapyridine in pneumococcic pneumonia. It was therefore necessary to study the susceptibility of animals to pneumonia on diets containing pantothenic acid and on pantothenate-free diets. The following report gives the results of this study.

Experimental

Two litters of albino rats were used. The first litter consisted of four males and three females with an average initial weight of 54.0 g.; the second had four males and four females and an average initial weight of 79.5 g. Seven rats, three from litter one and four from litter two, were placed on a basal synthetic diet of the following percentage

[1] Aided by a grant from the John and Mary R. Markle Foundation.

composition: casein,[2] 18; dextrose,[3] 68; hydrogenated cottonseed oil,[4] 8; salt mixture,[5] 4; cod-liver oil, 2. To each 100 g. of this ration was added thiamine hydrochloride,[6] 0.8 mg.; riboflavin, 0.8 mg.; pyridoxin hydrochloride, 0.8 mg.; nicotinamide, 10 mg.; choline chloride, 100 mg.; and calcium pantothenate, 0.9 mg. The remaining eight animals received the experimental diet which was of the same composition as the basal diet except that it contained no pantothenate. The animals were kept in raised-bottom cages and allowed food and distilled water *ad libitum* for 20 days. At the end of this period each animal received 0.2 cc. of a 1:1 mixture of an 8 per cent solution of mucin and a 24 hour infusion broth of the "Robertson" strain Type I pneumococcus by nasal insufflation.

Animals surviving a subsequent seven day period were sacrificed. Cultures of material from the lungs were made. Any gross pathology in the lungs was noted and portions of these organs together with the adrenal glands were removed for histological section.

Results

Average weight gains in litters one and two respectively were, for animals on the basal diet, 76.9 g. and 76.3 g., and for animals on the experimental diet, 43.4 g. and 29.0 g. The eight animals on the pantothenate-free diet showed coproporphyrin secretion on the nose and whiskers, roughening and thinning of the hair, marked reduction in the growth rates, and 6 developed adrenal cortical hemorrhagic necrosis.

One of the animals on the experimental diet succumbed three hours following the insufflation apparently as a result of the treatment. Three rats on the basal diet developed acute symptoms of pneumonia. One died[7] on the fourth day and the remaining two on the fifth day following insufflation.

Gross appearance of the lungs of the animals that died showed mas-

[2] Vitamin-free, "Labco."

[3] "C. P." Anhydrous. Mallinckrodt.

[4] "Crisco."

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.* **37**, 557 (1919).

[6] Crystalline B Vitamins were purchased from Merck and Company, Rahway, N. J.

[7] The three rats which died as a result of the infection showed massive hemorrhage into the adrenals. It is our opinion that this was probably the result of a generalized septicemia unrelated to pantothenic acid deficiency.

sive involvement; in those on the basal diet there were many points of consolidation as indicated by the hemorrhagic areas, and in one animal an entire side was involved; but the lungs of the rats on the pantothenate-free diet showed only occasional patches, and in some the lungs appeared nearly normal.

The animals that succumbed as well as the remaining animals from both basal and experimental groups had lung cultures which showed Gram-positive encapsulated diplococci. Microscopic sections from the lungs of the animals that died showed in the alveoli massive accumulation of exudate which was composed of erythrocytes, a few polymorphonuclear leucocytes and some mononuclear cells. Rats on the basal diet showed numerous patches of inflammation with exudate in the alveoli containing red blood cells and leucocytes. However, the histological sections of the lungs from every animal on the pantothenate-free diet showed only occasional patches of inflammation with exudate into the alveoli.

Discussion

The observations reported here appear to demonstrate that pantothenate-deficient animals are less susceptible to pneumococcic pneumonia than animals receiving the vitamin in the diet. The basal diet contained 0.9 mg. of calcium pantothenate, added, with the other crystalline vitamins, to 100 g. of food giving about 8.9 μg. of the nutrilite per gram of food. The average daily food consumption was 9.82 g. and thus the average amount of pantothenate ingested was 87.4 μg. of calcium pantothenate. A daily supplement of 100 μg. of calcium pantothenate represents approximately the optimum daily requirement (3). Of the seven animals receiving this diet 3 died as a result of acute pneumococcic pneumonia, and the remaining animals showed widespread pathology in the lungs in both gross and histological section. On the other hand, not a single animal on the pantothenate-free diet succumbed. Only one of these animals exhibited any clinical symptoms but they were not acute, and examination at autopsy and histological section showed only a small amount of involvement. Of the remaining 6 pantothenate-deficient animals, clinical symptoms, gross and microscopic appearance of the lungs, *invariably* showed less involvement than the controls. It is to be emphasized that the experimental animals were pantothenate-*deficient*,—not pantothenate-*free*. Consequently, *some* infection in these animals is a necessary and expected result. This work is being continued.

Summary

Pantothenate-deficient albino rats appear to be less susceptible to infection with type I pneumococcus than litter mates receiving the vitamin.

It is felt on the basis of this observation and our earlier demonstration of the inhibition of pantothenic acid by sulfapyridine in normal rats (1) that the mechanism of the action of this drug lies in its ability to inhibit in some way the pantothenic acid in the tissues and body fluids of the animals making it no longer available for the growth of the microorganism. Since the nutrilite is essential for the pneumococcus of the type used in these experiments, the growth is retarded giving the familiar picture of bacteriostasis.

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The Influence of Various Chemicals and Vitamin Deficiencies on the Excretion of Glucuronic Acid in the Rat

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Introduction

Certain aliphatic alcohols, aldehydes, ketones, terpenes, phenols, hydrocarbons, certain amines, aromatic acids, and many heterocyclic nitrogen compounds are detoxified by conjugation with glucuronic acid (1, 2, 3, 4). Many of the compounds thus detoxified are medicinal agents (5, 6, 7). It has been suggested that glucuronic acid might be used clinically to decrease the toxicity of sulfonamides (8) but the difficulty of preparing glucuronic acid in any degree of purity precludes the practical application of this procedure. It was therefore deemed advisable to seek a simpler chemical which would increase the formation of glucuronic acid in the body.

Procedure

Glucuronic acid (GA) content of the urine was determined by the use of naphthoresorcinol according to the technique of Maughan, *et al.* (9). As pointed out by Bueding (10) limited accuracy, is achieved in applying any naphthoresorcinol method for GA. Some substances (glucose, etc.) inhibit the color development; others (mucic acid) tend to increase it. To circumvent this lack of specificity in method, the positive experiments were checked using the method of Quick (11) and the newer method of Bueding (12). It is felt by the present authors that the napthoresorcinol method can be safely applied to a dilute medium such as urine. This is in accord with the reports of other groups (13, 14, 15, 10). Meyer, *et al.* (15) stated, "None of the methods available gave accurate results for uronic acids in native fluids, with the possible exception of normal urine, since carbohydrates and other compounds containing carbonyl groups as well as proteins and their decomposition products interfered." In the experimental studies here reported, if, for example, mucic acid was being tested for its effect on GA excretion, a check was made using a solution containing an amount of the mucic acid equal

to the amount of GA determined. If at this concentration, the mucic acid gave a significant color, the series was discarded. Glucurone, prepared by the method of Williams (16) was used as the standard for all determinations.

All other chemicals unless specified were chemically pure reagent grade. The animals were housed in metabolism cages and urine samples collected under toluene for 24 hour periods after the oral administration of the chemical under test. Glass wool and special filtering devices prevented contamination of the urine samples.

TABLE I

The Effect of Various Compounds on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hr. period)

Compound	Dosage	Glucuronic acid excretion					
Control	*g./kg.*	*80 mg./kg. Av. value for 15 sets of 12 rats each. Max. 95.2 mg./kg., Min. 73.4 mg./kg.*					
Dihydroxyacetone	4	129.0	104.5				
Glycerine	4	168.0	153.0	111.0	135.0		
Lactic Acid	2	117.0	98.0	119.0	140.0	127.0	91.0
Ca Glycerophosphate	4	109.0	60.0	44.0			
Pyruvic Acid	2	24	37	58	127	27	
Acetic Acid	2	55	105	81	118	58	
Glycolic Acid	2	65	101	85			
Na Ethyl Oxalacetate	2	62	92	73	11	96	
Tartaric Acid	2	129	53	41			
Succinic Acid	4	104	156				
Malic Acid	4	231	178				
Fumaric Acid	4	69	83				
Citric Acid	6	86	99	80	98		
Saccharic Acid	4	132	125				
Glucurone	4	94	105				
Ca Phytate	4	58					
Glycerine + Malic Acid	4 ea.	106					
Adenylic Acid + Malic Acid	0.5 & 4	210					
Adenylic Acid	0.5	134	158				
Nucleic Acid	4.0	92	108				

Each value represents the average for 10 sets of 2 rats in each set.

Results

Table I presents the results obtained with carbon compounds. Included in this table are adenylic acid and nucleic acid.

Dihydroxyacetone, glycerine, and lactic, succinic, saccharic, malic, and adenylic acids increase GA excretion in the urine. Pyruvic acid decreased excretion of GA. It is believed that acetic acid, calcium gly-

cerophosphate, glycolic acid, sodium ethyl oxalacetate, tartaric acid, and calcium phytate also decreased GA excretion, but variability of results prevents a final statement. Fumaric acid, citric acid, glucurone, and nucleic acid had little effect.

Of the amino acids tested, histidine, glycine, and alanine decreased GA excretion. Tyrosine and cysteine increased the excretion of GA. Cystine had no effect. Table II presents the results obtained. The results with ammonium chloride and sodium bicarbonate are included to show the effect of disturbance in alkaline acid balance.

TABLE II

The Effect of Various Amino Acids on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Compound	Dosage	Glucuronic acid excretion				
	g./kg.					
Histidine	2	56	76	54	74	
Cystine	4	94	63	94		
Glycine	8	73	35	60	48	51
Tyrosine	4	159*	130	151	190	141
Cysteine Hydrochloride	4	128	123			
Alanine	4	48	61			
Ammonium Chloride	2	46	61	55		
Sodium Bicarbonate	2	51	80	58	51	

* Rats receiving stock diet + 10% *l*-tyrosine.

Each value represents the average for 10 sets of 2 rats in each set.

With the exception of riboflavin deficiency, all the vitamin deficiency states resulted in a marked decrease in the excretion of glucuronic acid. Table III lists the results and includes a value showing the effect of 48 hour starvation on the excretion of the acid. All values presented are for rats taken in a stage of a given vitamin deficiency before they became moribund and while their food consumption was still adequate to prevent the intervention of the starvation factor.

With the sulfonamides, the results were variable. In acute experiments all tended to increased GA excretion excepting sulfapyridine. Sulfaguanidine was tried in a chronic experiment in which it constituted 2% of the diet. Under these conditions the rats after two months on the diet showed a marked drop in the excretion of the organic sugar acid.

TABLE III

The Effect of Various Vitamin Deficiencies on Glucuronic Acid Excretion

(Values expressed in mg./kg. of rat for a 24 hr. period)

Deficiency	Urinary glucuronic acid content					
Pantothenate	92	24	54			
B_1 Thiamine	29.9	45.8				
B_2 Riboflavin	114	39	77	107	103	127
D	21.9	33.8	48	105		
A	43.3	80	27			
E	2.3	17.7	15.2	44		
B_6 Pyridoxin	38	33				
Starved 48 hrs	20					

Each value represents the average of 10 sets of 2 rats each.

TABLE IV

The Effect of Various Sulfonamides on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Sulfonamide	Dosage	Added compound	Dosage	Glucuronic acid in urine				
	g./kg.		*g./kg.*					
Sulfanilamide	2				83	118	84	110
Sulfanilamide	2	Pyruvic Acid	4	76				
Sulfanilamide	1	Glucurone	2	80	198			
Sulfanilamide	2	Lactic Acid	4	75				
Sulfanilamide	2	Sodium Acetate	4	37				
Sulfanilamide	2	Malic Acid	4	94				
Sulfapyridine	4			59	31	28	34	
Na Sulfathiazole	1			210	134			
Sulfadiazine	6			97	60			
Sulfaguanidine	2% diet, 2 months			11.5	28.8			

Each value represents average for 10 sets of 2 rats each.

Tests conducted with sulfanilamide plus a compound known to produce an effect, positive or negative, on the excretion of GA showed that the sulfonamide tended to return all values to a more nearly normal figure.

Discussion

The objective of this work and of a companion effort (17) was to determine methods by means of which decreased acetylation and increased glucuronic acid formation could be brought about. The hope was that certain compounds might force the detoxication, particularly of sulfanilamide, away from acetylation and into combination with glucuronic acid. The literature contains ample evidence of the probability of this concept.

Our results *in vivo* in rats confirm those of Lipschitz and Bueding (18) *in vitro* using rat liver, in that dihydroxyacetone and lactic acid increased the formation of GA; but they differ in that we did not observe an increase following the administration of pyruvic acid, actually finding decreased excretion of GA. Quick (19) reported that lactic acid decreased GA formation in dogs, a point of dissimilarity with our findings in the rat. His result with glycolic acid is confirmed by our work. Another point of dissimilarity is in our observation that alanine decreased GA excretion, which would be expected if alanine were converted into pyruvic acid by oxidative deamination. Here, our results are in disagreement with both Lipschitz and Bueding (18) and Quick (19). Adeline (20) found that glycine, alanine, arginine, tyrosine, and histidine increased the output of glucuronic acid. In our experience alanine, glycine, and histidine decreased glucuronic acid formation, while tyrosine and cysteine increased its formation. It is probable that all seeming discrepancies observed are merely the result of dosage and species differences.

The sequence of potency with regard to stimulation of GA formation is in the order of decreasing effect: glycerine, dihydroxyacetone, and lactic acid. It is therefore suggested that the three carbon unit precursor sequence is from dihydroxyacetone phosphate to glycerophosphate and finally to phosphoglyceric aldehyde, which then condenses to a six carbon unit.

The phosphorylation of the precursors of GA was suggested by Lipschitz and Bueding (18). This work is corroborated by our observation that adenylic acid increased GA excretion. It is suggested that the high phosphate potential of adenylic acid aids in the phosphorylation of the three carbon unit precursor of GA. Further, this concept is strengthened by our observations that certain dicarboxylic acids, particularly succinic and malic, markedly increase the excretion of GA.

Kalckar (21) demonstrated in experiments with kidney extracts that the oxidation of dicarboxylic acids such as succinic, fumaric, and malic, gives rise to vigorous phosphorylation of various substrates. From the more recent work of Colowick, *et al.* (22) the conclusion is drawn that the generation of energy-rich phosphate originates with the dehydrogenation step from succinic to fumaric acid. It is therefore suggested that succinic and malic acids increase GA excretion in the rat by facilitating the phosphorylation of its three carbon unit precursor.

In riboflavin-deficient rats, the GA excretion is somewhat increased, this constituting the exception to the general rule of decreased excretion in various vitamin deficiency states. Riboflavin is involved in many enzyme systems, among these the dehydrogenation of malic acid in muscle, of lactic acid in muscle, etc. This might lead to increased concentrations of certain four carbon dicarboxylic acids and of certain three carbon units which lead to increased GA formation. The lactic acid oxidase system would be defective and prevent lactic acid, a compound stimulating GA production, from being oxidized to pyruvic acid, a compound inhibiting its formation.

It is much more difficult to explain the results seen with the other vitamin states. Liver pathology observed in rats in some deficiencies may explain defective GA synthesis.

Scudi and Robinson (23) have reported that sulfanilamide, in contrast to sulfapyridine and sulfathiazole, does not stimulate GA output. The work emphasizes the high percentage of sulfapyridine, some 40% of which is found in the form of a highly soluble glucuronide. This work and that of Weber, *et al.* (24) conclusively established the fact that sulfapyridine in the dog is followed by the appearance in the urine of a glucuronide of a hydroxy derivative of sulfapyridine. In our experiments, sulfapyridine clearly depressed GA excretion. It should be noted again that our experimental animals were rats, whereas Scudi and Robinson (23) and Weber, *et al.* (24) used dogs. Sulfanilamide, in our experience, increased to a slight degree the excretion of GA; sulfathiazole increased it to a marked degree; sulfadiazine had little if any effect; and sulfaguanidine fed chronically resulted in a profound decrease.

SUMMARY

Dihydroxyacetone, glycerine, lactic acid, succinic acid, malic acid, and adenylic acid markedly increase glucuronic acid excretion in the rat. Of the amino acids tested, only tyrosine and cysteine had a positive

effect. With the exception of riboflavin deficiency in which there is an increase in glucuronic acid excretion, all the other deficiency states showed decreased excretion of this acid. The effect of sulfonamides on the excretion of glucuronic acid by the rat was studied.

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Environmental Temperature and Protein Requirement

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Introduction

On account of their high specific dynamic action, proteins are usually favored in cold weather diets and avoided in the heat. Our recent demonstration of heightened thiamine and choline requirements in tropical warmth (1) necessitates a reconsideration of the matter, however, for most natural food sources rich in the B vitamins are also high in protein. Meats and other animal products (eggs, milk, cheese, etc.) are particularly important sources for these vitamins, containing them in relatively large amounts and in a better balance than is found in the cereal grains. Waisman and Elvehjem (2) emphasized these points in their recent monograph.

It would be unfortunate, therefore, if heat loss difficulties should call for reduced dietary protein in hot weather when the individual needs increased amounts of the vitamins normally carried in protein-rich foods. Since hot weather avoidance of proteins seems to have been based entirely upon theoretical grounds, it was decided to subject the matter to experimental test.

Experimental

Weanling male white rats were placed two to the cage (in groups of 4) in the two rooms previously described (3), one room being kept at 90–91°F. and 60–70% relative humidity and the other at 68°F. Weekly estimates were made of food consumption and weight gain. The diet used for Group I was composed as follows:

Casein (S.M.A., vitamin-free)	6 g.	per	100 g.	of	diet
Sucrose	88 "	"	"	"	"
Corn oil	2 "	"	"	"	"
Salts, as previously described (1)	4 "	"	"	"	"

Note: The B vitamins used in this study were very kindly supplied by Merck & Co., Inc.; and the Haliver oil by the Abbott Laboratories.

Haliver oil	1.2 cc.	per 1000 g. of diet
Thiamine-HCl, in the cold room	1 mg.	" " " "
in the hot room	2 "	" " " "
Riboflavin	4 "	" " " "
Pyridoxin	4 "	" " " "
Calcium pantothenate	6 "	" " " "
Nicotinic acid	25 "	" " " "
Inositol	1 g.	" " " "
p-Aminobenzoic acid	0.3 "	" " " "
Choline, in the cold room	0.75 "	" " " "
in the hot room	5.0 "	" " " "

For the succeeding four groups in each room the casein content of the diet was adjusted upward from 6 to 12, 18, 24, and 36 per cent respectively, with corresponding reductions in sucrose. With 4 such series of

TABLE I

Growth and Food Consumption in Heat and Cold at Various Levels of Dietary Protein

	At 68°F.					At 90-91°F. and 60-70% R.H.				
Per cent protein in diet	6	12	18	24	36	6	12	18	24	36
Food eaten (g's) during 3rd, 4th, 5th weeks on diet	158	247	302	300	295	71	128	156	205	215
Weight gain (g's) during 3rd, 4th, 5th weeks on diet	14.00 ±0.99	49.69 ±1.63	72.19 ±1.13	68.12 ±2.36	80.00 ±1.91	0.42 ±0.74	17.81 ±1.21	54.38 ±2.02	68.75 ±1.47	78.13 ±1.07
Final weight (g's) after 5 weeks on diet	70.00 ±1.66	130.00 ±2.21	171.87 ±1.86	170.00 ±3.86	180.00 ±2.53	46.67 ±1.58	85.00 ±2.15	142.50 ±2.02	170.00 ±2.38	176.88 ±1.91

rats (making 16 at each protein level in hot and cold), group differences in food consumption, weight gain, and final weight are shown in Table I.

Growth and food consumption were found to be optimal at the 18% protein level for the rats kept at 68°F., but at 91°F. best performance occurred with higher dietary protein. Differences in rates of gain and in final weights are marked and of high mathematical significance up to the 18% protein group in the cold and to the next higher group in heat. Amounts of food eaten could be estimated less accurately and are given only as average values.

Among the several series of rats observed (some for as long as 10 weeks), growth was most rapid in the heat with 36% protein during the first few weeks; later those on the 18% diet had come up to an optimal state of growth. At 68°F. there was little difference in growth during the early weeks at protein levels from 18% upward; later those on 12%

protein achieved almost optimal growth, while those on 36% protein lagged behind. This higher protein tolerance in young animals had previously been described by Smith and Moise (4).

Since casein is known to be low in cystine, and since this amino acid is so important in the body economy, it was thought that perhaps its addition to the rat diets might eliminate the apparent heightening of protein need in hot environments. Therefore another series of rats was placed in the rooms on the same diets with added cystine (2 g./kilogram). Weight gains for the last 3 weeks on the diets and final weights at the end of the 5-week period are shown in Table II.

Addition of 0.2% cystine to all diets brought about a very obvious improvement in the growth of all rats on suboptimal percentages of

TABLE II

Effect of Added Cystine upon Protein Requirement for Optimal Growth in Heat and Cold

	At 68°F.				At 90–91°F. & 60–70% R.H.			
Per cent protein in diet	12	18	24	36	12	18	24	36
With no added cystine:								
Weight gain 3rd, 4th, 5th weeks	50	72	68	80	18	54	69	78
Mean weight after 5 weeks on diet	130	172	170	180	85	143	170	177
With 0.2% added cystine								
Weight gain 3rd, 4th, 5th weeks	69	82	80	92	50	76	78	73
Mean weight after 5 weeks on diet	172	181	180	187	125	174	178	180

protein, this bettered growth being especially marked in the heat. Growth in both heat and cold now became practically optimal with 18% dietary protein. Although a slightly higher dietary percentage of protein seems needed in the heat, daily food consumption is 30% lower there than in the cold. Each unit of growth is thus accomplished with fewer calories and fewer grams of protein intake in the heat. These results in a way verify those of Treichler and Mitchell (5), although their period of cold adaptation (only one week) was much too brief for full metabolic response.

Conclusions

After proper correction is made for the heightened requirement for thiamine and choline in tropical heat, just as good growth can be obtained in the heat as in the cold. Slightly higher percentages of dietary

protein are needed in the heat, however, although the total daily protein intake is actually lower.

Fortification of the diet with cystine (0.2%) lowers the amount of protein needed for optimal growth in both heat and cold, and it seems to eliminate the need of a higher protein percentage for optimal growth in the heat.

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A Comparison of the Chemical Protein Quality Index with the Gross Protein Value of Fish Protein Concentrates[1]

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Introduction

Several methods for estimating the nutritional value of protein supplements have been devised. Those used most commonly are some form of the nitrogen balance method or the growth and weight maintenance method. The latter is the one most used in evaluating protein supplements for poultry. There is no standard method of making this estimation, the different procedures differing in level of protein in the diet, length of experimental period, and feed consumption. A number of these methods were reviewed by Heiman, Carver, and Cook (2). St. John, Johnson, Carver, and Moore (1) devised a method for making nitrogen balance studies with poultry.

A chemical method of estimating the nutritional value of animal protein concentrates for poultry was developed by Almquist, Stokstad, and Halbrook (3). They reported a good correlation between their chemical protein quality index and the growth of chicks for six weeks. Almquist (4) later compared the chemical protein quality index with the gain per gram of feed consumed by chicks from 4 to 6 weeks of age. Olson and Palmer (5) compared the chemical protein quality indices of several proteins with their biological value as determined by nitrogen-balance studies with rats. They reported variable results on isolated proteins but obtained a close correlation using natural foods.

In view of the wide variation between different methods of estimating the nutritional value of protein concentrates for poultry observed by

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Cook, St. John, and Heiman (6), it appeared advisable to further compare the chemical protein quality index (3) with a growth and weight maintenance method. Heiman, Carver, and Cook (2) developed such a method based on the gain in weight per unit of supplementary protein when a low level of protein was fed. The value obtained when compared with casein as 100 was termed the gross protein value.

Experimental

Seven samples of experimentally prepared fish meals were obtained and the gross protein values determined as a part of another experiment.[2] These consisted of two samples of pilchard fish meal and five samples of dogfish meal (samples 719 to 725 inclusive). The methods of preparation are indicated in Table I. The gross protein values of commercial samples of herring fish meal, pilchard fish meal, casein, and meat scrap also were determined.

The gross protein values were determined as outlined by Heiman, Carver and Cook (2). Day-old chicks were fed for two weeks a cereal and sucrose depletion diet containing 8 per cent protein. They then were divided into groups containing 15 chicks per group. The experimental diets contained 11% protein. 3% of this was furnished by the protein supplement under investigation, which replaced an equal weight of sucrose. Each diet was fed to duplicate groups of chicks. Two groups were continued on the depletion diet as negative controls. Gain in weight and feed consumption records were kept. From the results, the increase in body weight of the experimental chicks over the negative controls, and the gain in weight per gram of supplementary protein were calculated. Rather than using the gross value (obtained by comparison with casein), the gain in weight per unit of supplementary protein fed was used.

The gross protein value of a combination of 25% powdered feather protein and 75% soybean oil meal protein was determined. The feathers were prepared by grinding them in a Wiley Mill and then grinding for several days in a ball mill. The soybean oil meal by itself gave a gain of 6.4 g. per gram of supplementary protein, and the feather-soybean oil meal mixture gave one of 5.0 g. Assuming no supplementary action between the feather and soybean oil meal proteins, the grams of gain per unit of supplementary protein for the feathers was 0.8 g.

[2] These samples of fish meal were prepared by the U. S. Fish and Wildlife Service, Technological Laboratory, Seattle, Washington.

TABLE I

Comparison of the Chemical Protein Index and the Gain per Gram of Supplementary Protein for Fishmeals and Other Animal Protein Concentrates

Sample no.	Kind protein concentrate	Method of preparation	Gain per Unit Protein *g.*	Crude Protein %	Percent of total protein: Copper Pptn.	Phosph-tung.	Undi-gest.	Hot-water sol.	Protein quality index
719	Pilchard	None	7.8	62.0	94.8	1.9	8.4	6.6	83.0
720	Pilchard	Antioxidant	7.9	60.0	94.5	1.3	8.3	5.7	83.5
721	Dogfish	CH_2O and Acid	7.3	70.8	85.0	1.4	7.1	4.4	77.1
722	Dogfish	Wet process—low temperature	7.8	73.2	77.7	3.8	4.8	6.3	70.6
723	Dogfish	Wet process—low temperature. Antioxidant	7.3	69.2	79.1	3.2	5.2	7.1	71.2
724	Dogfish	Wet process—high temperature	7.0	70.2	77.6	3.8	5.3	6.3	70.2
725	Dogfish	Dry process—low temperature	5.7	82.1	50.3	11.4	3.8	10.2	44.5
703	Herring	Commercial	8.3	75.2	91.5	1.6	8.6	5.2	80.5
542	Pilchard	Commercial	8.4	71.6	93.9	1.4	8.1	5.0	83.5
677	Casein	Commercial	9.0	87.7	94.9	1.4	8.0	1.6	86.5
579	Meat	Commercial Scrap	2.6	57.5	62.8	27.0	8.0	30.3	47.5
760	Feathers	Powdered in ball mill	0.8*	81.8	98.9	0.9	81.6	2.3	14.9

* This value was a calculated value determined from a value of 6.4 g. per gram of supplementary protein for soybean oil meal and one of 5.0 g. for a mixture of 25 per cent feathers and 75% soybean oil meal.

The protein quality index was determined on the same samples by the method of Almquist, Stokstad, and Halbrook (3). This method consists essentially of a determination of the per cent of crude protein precipitated by $CuSO_4$, $Al_2(SO_4)_3$, and MgO; the per cent of crude protein precipitated by phosphotungstic acid; the per cent of crude protein not digested by pepsin; and the per cent of crude protein soluble in hot water and precipitated by $CuSO_4$, $Al_2(SO_4)_3$, and MgO.

The Cu-precipitable protein is intact protein. This fraction decreases as decomposition increases. The phosphotungstic acid precipitable protein is composed of peptones, peptides, and amino acids not precipitable by Cu. The undigestible protein fraction is composed of keratins, denatured proteins, and certain insoluble nitrogen compounds. The hot-water-soluble protein is composed mainly of gelatin.

The protein quality index was calculated by subtracting from the Cu-precipitable protein all of the undigestible protein and 0.6 of the hot-water-soluble protein and adding to the result 0.4 of the phosphotungstic acid-precipitable protein. The final sum was divided by the total crude protein and multiplied by 100. This formula was developed by Almquist, Stokstad, and Halbrook (3) as the one best correlating growth and chemical data for many types of proteins.

Results

The results are presented in Table I and in Fig. 1. The dogfish meals, for the most part, contained less Cu-precipitable protein and more phosphotungstic-acid-precipitable protein than the pilchard fish meals. The dogfish meal prepared at a low temperature by the dry process had an especially low Cu-precipitable protein fraction and high phosphotungstic acid precipitable and hot-water-soluble protein fractions.

A very high coefficient of correlation of 0.933 was obtained between the protein quality index and the gain per unit of supplementary protein fed for the seven experimentally prepared fish meals. A correlation of 0.874 was required to be highly significant. A coefficient of correlation of 0.924 was obtained when the commercial herring and pilchard fish meals were included in the calculation. A coefficient of 0.798 was required to be highly significant for the nine samples used.

The sample of meat scrap had a poor protein quality index and also gave poor growth. The low protein quality index was the result of a small Cu-precipitable protein fraction, and large phosphotungstic-acid-precipitable and hot-water-soluble protein fractions. The powdered

feathers gave the poorest results both in growth and protein quality index. Over 80 per cent of the crude protein of the feathers was not digested by pepsin.

A coefficient of correlation of 0.944 was obtained when all twelve of the animal protein concentrates were considered. To be highly significant a correlation coefficient of 0.708 would be necessary. The reason for the high correlations is evident from Fig. 1.

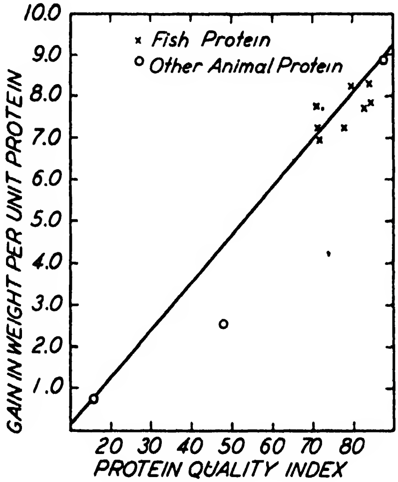

FIG. 1

Comparison of the Chemical Protein Quality Index with the Gain in Weight per Unit of Supplementary Protein for Fishmeals and Other Animal Protein Concentrates

The casein sample used in this experiment has given a very high value for the grams of gain per gram of protein consumed every time it has been used. It has given a value of 9.0 g. compared to values of 7.0 to 8.0 g. usually obtained.

DISCUSSION

The very good correlation obtained in this study between chemical and biological methods of determining protein quality supports the findings of Almquist, Stokstad, and Halbrook (3), Almquist (4), and Olson and Palmer (5). It gives further evidence of the value of the chemical method for estimating the nutritional value of animal proteins, particu-

larly fish meal proteins. Casein also fits closely into this relationship (see Fig. 1), differing from the results of Olson and Palmer (5).

The gross protein method (2) employed in this study differs from most other growth and weight maintenance methods because the level of protein was considerably below the optimum level for chicks. Total crude protein in the diets was 11% and that supplied by the protein supplement was 3%. This contrasts with the 19 and 15% total protein and 7 and 6% supplementary protein fed by Almquist and co-workers (3, 4), who also observed a good correlation between the biological and chemical methods.

An advantage of the chemical method over biological methods is that it offers an explanation of why one protein supplement is better than another. For example, the powdered feathers were of little value

TABLE II

Protein Distribution in Dogfish Meals

Treatment	Cu-ppt.	Phosphotungstic Acid-ppt.	Undigestible	Hot Water Soluble	Total Crude Protein
	%	%	%	%	%
Wet process, low temperature	56.9	2.8	3.5	4.6	73.2
Dry process, low temperature	41.3	9.4	3.1	8.4	82.1

because 81.6% of the protein was undigestible. The poor results here with powdered feathers contrast with the good results obtained by Wagner and Elvehjem (7) with powdered swine hoofs. The meat scrap was a poor protein supplement. The poor quality can be explained as being due to two factors: 30.3% of the protein was hot-water-soluble protein, probably gelatin which has a poor nutritive value; and only 62.8% of the crude protein was precipitated by Cu, while 27.0% was precipitated by phosphotungstic acid showing that considerable of the protein had disintegrated to peptones, peptides, and amino acids.

The chemical values obtained for the different fish meals were of special interest since work has been in progress on the nutritional value of fish meals for several years at this station. Recently Rhian, Carver, Harrison, and Hamm (8) found dogfish meals prepared by the wet process to have much higher nutritive values than similar meals prepared by the dry process. The results presented in Table II offer one explanation of the differences. The dry process meal, although containing 8.9% more

protein than the wet process one, contained 15.6% less Cu-precipitable protein. Part of this was accounted for in the phosphotungstic-acid-precipitable protein fraction, but there was more nitrogen unaccounted for in the dry process meal than in the wet process one. The dry process meal also contained more hot-water-soluble protein. There appears to have been more decomposition occurring in the dry process meal than in the wet, much of it going to the amino acid stage or further. Considerable of the hot-water-soluble proteins fraction appears to have been removed by the wet process method of preparation.

Summary

The chemical protein quality index and the gross protein value were determined on seven samples of experimentally prepared fish meal, two of commercial fish meal, one of commercial meat scrap, one of commercial crude casein, and one of powdered feathers. There was a positive coefficient of correlation of 0.944 between these two values for the 12 animal protein samples. A correlation coefficient of 0.933 for the seven experimental fish meals and one of 0.924 for all of the fish meals gives evidence of the value of the chemical method for estimating the nutritional value of fish meals. Dry process dogfish meal was inferior to wet process dogfish meal by both methods of estimation.

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Phenol Studies
V. The Distribution, Detoxification, and Excretion of Phenol in the Mammalian Body

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Introduction

The metabolism of phenol has been made the subject of many an investigation, but it appeared desirable to reinvestigate certain phases of this problem inasmuch as accurate quantitative methods for the separate estimation of "free" and "conjugated" phenol in organs, tissues, and fluids (10), and also a quantitative method for the estimation of phenol-glucuronate in urine (11) have become available only recently. The following paragraphs offer a brief review of the literature and summarize the experimental results on (A) the distribution of phenol in the normal animal; (B) the distribution of phenol in the organs and excreta of animals after oral poisoning; and (C) the rate of excretion, conjugation, and breakdown of phenol in rabbits and rats poisoned with this compound.

Review of the Literature

In a previous article (10) a review was given of the quantities of phenol found in blood and urine by earlier investigators, and also the results of our studies on the "free," "conjugated," and "total" phenol content of normal human organs, blood, urine, saliva, sweat, and feces.

That the body has three general mechanisms for the disposal of phenol —(a) conjugation with sulfuric and other acids, (b) oxidation, and (c) excretion of the unchanged material—was established in 1876, particularly by Baumann (4) but in part also by Schmiedeberg (24), Salkowski (22), Hoppe-Seyler (16), and others. In the same year, Baumann made

another most important contribution when he isolated phenol-potassium sulfate from the urine of horses. He also synthesized this compound and administered 2.6 g., equivalent to 1.1 g. phenol, orally to a rabbit. When he saw that this dose produced no signs of illness, he concluded that phenol-potassium sulfate represented a detoxified form of phenol. Christiani (8, 9) repeated Baumann's feeding experiment and found that 72% of this salt (0.59 g./kg.) was excreted unchanged in the 24 hours after administration.

Quantitative data on the amounts of phenol disposed of by oxidation were reported by Tauber (27), Baumann and Preusse (7), and by Nencki and Giacosa (20). They found that in experimental animals from 25 to 50% of sublethal doses were broken down to carbon dioxide and water, while traces were oxidized to pyrocatechol and hydroquinone.

Much work has been done to determine the site of the detoxification of phenol; the data are somewhat conflicting, but it has been definitely established that the liver plays the most important rôle; however, the kidney and other organs, even the epithelial lining of the intestinal tract, and the blood, appear to take part in this function (Baumann, 5, 6; Herter and Wakeman, 15; Marenzi, 19; Barac, 1, 2, 3; Embden and Glaessner, 14; and Lipschitz and Bueding, 18). In dogs poisoned by phenol, Satta (23) found the highest concentration of "conjugated" phenol in the liver, with the kidneys, stomach, intestine, lungs, and blood following in this order. That the liver plays a predominant rôle is also indicated by the fact that Pelkan and Whipple (21) found the minimal lethal dose of phenol to be markedly lower in dogs whose livers had been injured prior to treatment with phenol.

It has been demonstrated that excretion of "free" phenol in the urine plays no great rôle in the disposal of those amounts normally formed in the body; but various authors have stated that when sublethal doses of phenol are administered to animals, approximately 30 per cent of the total amounts absorbed is excreted in the "free" form during the first 24 hours (Christiani, 8, 9; Tauber, 27; Jonescu, 17; Siegfried and Zimmermann, 25, 26; Ellinger, 13; Duce, 12).

Experimental Procedures

Albino rabbits (2–3 kg.) were purchased from a local breeder, and kept under observation for at least 2 weeks before being used; they were fed Purina Rabbit Pellets. The rats, from a Wistar Institute colony, were

born and reared in the laboratory; their diet consisted of Purina Fox Chow.

Phenol, Merck's reagent grade, was administered to the rabbits as a 5 per cent aqueous solution by means of a stomach tube. The rats were given a 0.5 per cent aqueous solution in a similar manner.

All analyses were carried out by a spectrophotometric method (10) by which "free" and "conjugated" phenol are determined separately in tissues and fluids. The total phenol was obtained by summation of these fractions. The urinary sulfates were estimated by the turbidimetric method of Treon and Crutchfield (28), and urinary glucuronic acid by a quantitative procedure described elsewhere (11).

Results

(A) The Distribution of Phenol in Normal Animal Tissues

The blood and tissues of 6 rabbits were found to contain either no phenol or only insignificant traces; the figures were within the same limits as those determined for normal human material. With two exceptions, there were approximately equal proportions of "free" and "conjugated" phenol; the central nervous system contained no "free" phenol at all, while the bulk of phenol found in the liver was conjugated. The 24-hour urine samples contained from 0 to 0.39 mg. "free," from 1.15 to 10.0 mg. "conjugated," and from 1.15 to 10.0 mg. of total phenol (Table I).

The concentration of phenol in the carcasses of six 100 g. rats ranged from 0.17 to 0.36 mg. "free," from 0.22 to 0.37 mg. "conjugated," and from 0.4 to 0.6 mg. total phenol. The 24-hour samples of both urine and feces contained about 0.25 mg. total phenol, the bulk being conjugated.

(B) The Distribution of Phenol in the Body of the Rabbit after Poisoning with This Compound

Table II shows the distribution of phenol in the organs and tissues of 5 rabbits given a lethal dose (0.5 g./kg.), orally. The animals were killed by air embolism when the first twitching appeared—in from 1 to 3 minutes. It is of interest to note that in this short period of time, a) phenol had found its way into all the tissues; b) the largest amounts were found in the liver and the central nervous system, although the lungs and blood

also contained large amounts; and c) the process of detoxification by conjugation had definitely started, as indicated by the elevated amounts of "conjugated" phenol in the liver and kidneys.

TABLE I

Phenol Content of Normal Rabbit Tissues

(6 animals)

Tissue	Phenol (mg./100 g.)		
	Free	Conjugated	Total (*by summation*)
Blood	0–0.07	0– 0.05	0– 0.07
Central Nervous System	0	0– 0.18	0– 0.18
Kidney	0–0.10	0– 0.05	0– 0.14
Lung	0–0.23	0– 0.34	0– 0.34
Liver	0–0.09	0.11– 0.55	0.11– 0.62
Muscle	0–0.16	0– 0.18	0– 0.34
Gastroenteric Tract Plus Contents	0–0.30	0– 0.23	0– 0.44
Heart, Spleen, Thymus, Testes, Adrenals	0–0.03	0– 0.10	0– 0.10
Urine (24 hr. volume)	0–0.39	1.15–10.00	1.15–10.00
Feces (24 hr.)	0.04–0.53	0.14– 0.80	0.18– 1.17

TABLE II

Distribution of Phenol in the Organs of Five Rabbits Killed 1 to 3 Minutes after an Oral Dose of 0.5 g. Phenol per Kilogram of Body Weight

Tissue	Phenol (mg./100 g.)		
	Free	Conjugated	Total (*by summation*)
Liver	19.2–29.3	1.0–3.1	20.9–30.4
Lungs	4.7–16.7	0.4–0.8	5.1–17.1
Blood	5.3–10.9	0.3–0.9	6.2–12.6
Brain and Cord	2.9–10.0	0–0.4	3.1–10.4
Kidneys	2.0– 6.0	0.2–1.0	2.3– 7.1
Muscle	0.3– 3.8	0–0.4	0.8– 3.8
Urine (taken from bladder)	0– 0.1	1.6–2.9	1.7– 2.9

To each of another group of 5 rabbits, a corresponding oral dose was administered, but the animals lived for from 15 minutes to 6 hours (some died, others were killed). Their tissues were analyzed individually as

TABLE III

Distribution of Phenol in the Organs of Rabbits That Died or Were Killed within 6 Hours after an Oral Dose of 0.5 g. of Phenol per Kilogram of Body Weight

Tissue	Phenol	Died after 15 min.	Died after 82 min.	Killed after 2 hrs.	Killed after 2½ hrs.	Killed after 6 hrs.
		Concentration of Phenol in milligrams per 100 g. of Tissue				
Liver	Free	63.7	22.4	3.4	13.5	0.5
	Conjugated	0.9	4.2	3.2	6.0	9.4
	Total**	64.6	26.6	6.6	19.5	9.9
Blood	Free	30.8	22.4	5.8	11.3	6.5
	Conjugated	0.9	5.3	8.0	10.2	9.8
	Total	31.7	27.7	13.8	21.5	16.3
Kidneys	Free	35.3	13.4	4.8	11.2	2.6
	Conjugated	0.8	7.4	22.8	12.9	30.0
	Total	36.1	20.8	27.6	24.1	32.6
Lungs	Free	34.2	20.8	5.4	12.2	1.5
	Conjugated	1.8	4.7	6.7	5.1	3.0
	Total	36.0	25.5	12.1	17.3	4.5
Heart, Thymus, Testes, Spleen	Free	53.0	21.0	6.8	14.0	7.5
	Conjugated	0.6	2.3	5.7	5.1	7.7
	Total	53.6	23.3	12.5	19.1	15.2
Brain and Cord	Free	31.3		6.8	10.4	2.5
	Conjugated	0.5		0.7	0.3	0.4
	Total	31.8		7.5	10.7	2.9
Muscle	Free	19.0	8.2	9.2	12.0	10.1
	Conjugated	0	0.5	1.1	0.8	1.4
	Total	19.0	8.7	10.3	12.8	11.5
Urine	Free		0.5		11.6	11.0
	Conjugated	no sample	14.0	no sample	52.0	12.3
	Total		14.5		63.6	23.3
Exhaled Air	Free	0	0.1*	0.7*	0.1*	0.2*
	Conjugated					
	Total	0	0.1	0.7	0.1	0.2

* Phenol in total air exhaled.

** Total phenol obtained by summation of free and conjugated fractions.

in the previous cases. The data are summarized in Table III which shows that a) the largest amount of ingested phenol was recovered from the organs and tissues of the rabbit that died in 15 minutes, the smallest amount from the animal that lived 6 hours; b) during the period from 15 minutes to 6 hours, the concentration of "free" phenol in all organs and tissues decreased while that of the "conjugated" phenol increased; c) on the basis of the total weight or bulk of the organs and tissues, the muscle tissue contained from 3.4 to 7.7% of the ingested phenol, while the liver and total volume of blood furnished nearly equal amounts, about 2% each; and d) traces of phenol were eliminated in exhaled air.

(C) The Detoxification and Excretion of Phenol in Rabbits and Rats Poisoned by Oral Doses of the Compound

The detoxification and excretion of phenol was studied following the oral administration of sublethal and lethal doses to rabbits and rats. As might be expected, the results are quite variable. Table IV shows the extent of the recovery of phenol from the carcasses of the rabbits at the end of 24 hours (at which time the animals were killed), and from the excreta collected during this period following the administration of half a lethal dose. The difference between the quantity administered and that recovered in the individual case may be presumed to be the quantity destroyed in the body of the animal.

From these data it is evident; (a) that in the rabbit roughly 77% of a toxic (half lethal) dose is excreted in the urine and only traces are excreted in the feces within the first 24 hours; (b) that about half of the excreted phenol is "free" and the other half "conjugated"; (c) that roughly 20% is destroyed; and (d) about 3% of the amount ingested is still present in the body at the end of the first day. The type of conjugation which phenol undergoes when administered to rabbits in these concentrations is shown in Table V, in which it may be seen that of the total amount of phenol excreted in the "conjugated" form, roughly 18% is conjugated with glucuronic acid, about 53% with sulfuric, and approximately 29% with acids not yet identified. (All glucuronates and ethereal sulfates were assumed to be in combination with phenol.)

The recovery of phenol from the carcasses and excreta of rabbits that lived from 15 minutes to 6 hours, following the oral administration of a lethal dose of phenol, is summarized in Table VI. These data show (a) that only traces of phenol were excreted in the urine (the result of an almost complete anuria); (b) that about 85% of the phenol excreted in

the urine was conjugated; (c) that roughly 50% of the ingested phenol was destroyed in from 2½ to 6 hours; and (d) that the other half of the dose was recovered from the carcass.

TABLE IV

Recovery of Phenol from the Bodies and Excreta of Rabbits 24 Hours after the Oral Administration of a Sublethal Dose of 700 mg.

(About 0.3 g. Phenol per Kilogram of Body Weight)

Identification number of rabbit	Phenol recovered in urine*		Phenol recovered in feces*		Phenol found in carcass		Phenol destroyed (by difference)
	mg.	*% of amount administered*	*mg.*	*% of amount administered*	*mg.*	*% of amount administered*	*% of amount administered*
10	412	58.6	4	0.6	21	3.0	37.8
11	591	84.4	4	0.6	20	2.9	12.1
12	615	87.8	3	0.4	16	2.3	9.5
13	545	77.8	2	0.3	33	4.7	17.2

* Approximately half was "free," the other half "conjugated."

TABLE V

Type of Conjugation Undergone by Phenol in the Rabbit Following Oral Administration of a Sublethal Dose of 0.3 g. of This Compound per Kilogram of Body Weight

Identification number of rabbit	"Conjugated" phenol excreted in urine during the first 24 hours *mg.*	Phenol conjugated with glucuronic acid		Phenol conjugated with sulfuric acid		Phenol conjugated with acids not yet identified	
		mg.	*% of total amt. conjugated*	*mg.*	*% of total amt. conjugated*	*mg.*	*% of total amt. conjugated*
1	315	157	49.8	96	30.4	62	19.8
2	247	38	15.4	167	67.6	42	17.0
3	261	8	3.1	180	68.9	73	28.0
4	258	33	12.7	110	42.6	115	44.7
5	267	29	10.9	141	52.8	97	36.3

From these experiments it is apparent that when phenol is administered in sublethal doses to rabbits, detoxification by conjugation is the major means by which the compound is eliminated from the body of the

TABLE VI

Recovery of Phenol from the Bodies and Excreta of Rabbits Following Oral Administration of a Single Lethal Dose of 0.5 g. Phenol per Kilogram of Body Weight

Time interval between administration and death	Amount of phenol administered per animal	Phenol recovered in urine and feces		Phenol found in carcass		Phenol destroyed (by difference)
	mg.	*mg.*	*% of amount administered*	*mg.*	*% of amount administered*	*% of amount administered*
Died after 15 minutes....	1750	0	0	1340	76.5	23.5
Died after 82 minutes ...	1950	14.5	0.7	1449	74.3	25.0
Killed after 2 hours ...	1410	0	0	970	68.8	31.2
Killed after 2½ hours. ..	1340	63.6	4.7	729	54.4	40.9
Killed after 6 hours... ..	1550	23.3	1.5	679	43.8	54.7

TABLE VII

Destruction and Recovery of Phenol from the Bodies and Excreta of Rats Following Oral Administration of a Sublethal or Approximately Lethal Dose of Phenol

Time interval between administration and death	Dose administered		Phenol in total carcass and excreta*			Phenol destroyed (by difference)	
	g./kg.	*mg./animal*	*"free"*	*"conjugated"*	*total*	*mg.*	*% of amount ingested*
30 minutes	0.2	12	6.3	4.1	10.4	1.6	13.3
2 hours...........	"	12	1.6	1.6	3.2	8.8	73.3
4 hours...........	"	12	0.9	1.3	2.1	9.9	82.5
30 minutes.........	0.4	30	17.9	1.9	19.8	10.2	34.0
30 minutes	"	34	20.8	0.5	21.3	12.7	37.4
2 hours......... ..	"	28	11.5	1.7	13.2	14.8	52.8
4 hours......... ..	"	36	11.2	3.7	15.9	20.1	55.8
4 hours............	"	30	5.4	4.6	10.0	20.0	66.0
30 minutes........	0.6	45	34.9	0.3	35.2	9.8	21.8
2 hours............	"	45	27.0	5.0	32.0	13.0	29.0
4 hours............	"	45	17.6	6.4	24.0	21.0	46.6
4 hours............	"	45	7.1	2.2	9.3	35.7	79.3

* The excreta contained only traces of phenol and therefore the results have been combined with those obtained on the carcasses.

animal. However, when the administered dose is of approximately lethal proportions (and the excretion of the conjugated product is diminished as the result of injury to the kidneys), the destruction of the compound by oxidation is the predominant mechanism.

Experiments were then conducted on rats in order to determine whether the same processes occurred in this species. Sublethal and approximately lethal doses were also administered to these animals, which then died or were killed at various intervals up to and including 4 hours after the administration. The carcass, the urine, and the feces of each animal were analyzed separately, but since the urine and feces contained only traces of phenol the results are combined under one heading in Table VII. The analytical data show that oxidation was well under way in every case in 2 hours. At the end of 4 hours the percentages of oxidized phenol were roughly 80% in the case of rats given 0.2 g./kg., and 60% in the case of those to which 0.4 or 0.6 g./kg. were administered.

Summary

1. The tissues of normal rabbits and rats contain insignificant traces or no "free" or "conjugated" phenol. The compound is present in urine in larger amounts, the bulk being in the "conjugated" form (Table I).

2. In animals poisoned with phenol, the compound penetrates all the tissues with extreme rapidity and the severity of the intoxication is in direct relation to the concentration of "free" phenol in blood and tissues. Phenol is removed from the animal body by excretion, oxidation, and conjugation; the latter two processes apparently start before symptoms of poisoning appear. The proportion of the total dosage that is disposed of in each of these three ways varies with the size of the dose administered and with the animal species employed experimentally (Tables II and III, VI and VII).

3. The fate of half a lethal dose of phenol, during the first 24 hours after its oral administration to rabbits, is as follows:

Roughly 77% (59 to 88%) of the ingested material is excreted in the urine. Approximately half of this amount is present as "free" phenol; of the "conjugated" fraction, about half is conjugated with sulfuric acid, a little more than one-fourth with glucuronic acid, and the remaining portion is conjugated with other acids not yet identified.

Roughly 20% (10 to 38%) of the ingested material is destroyed. (According to earlier investigators the bulk is oxidized to carbon dioxide

and water, and small amounts to pyrocatechol and hydroquinone. Traces of the last two compounds are broken down in the body to dark colored substances, traces are excreted unchanged with the urine, while the bulk is conjugated and then excreted with the urine. On exposure to air the conjugated compounds hydrolyze; pyrocatechol and hydroquinone are further changed into colored substances which give the urine its "smoky" appearance.)

Traces of "free" and "conjugated" phenol are excreted in the feces. Faint traces of "free" phenol are eliminated in the exhaled air. From 2 to 5% of the ingested material is still present in the carcass at the end of the first day.

4. The fate of a nearly lethal dose of phenol, during the first few hours after its oral administration to the rabbit, is as follows:

About 48% (41 and 55%) of the ingested material is destroyed, and about 50% (55 and 44%) is still present in the carcass approximately 4 hours afterward. After 2½ hours the larger proportion is present as "free" phenol, after 6 hours it is in the "conjugated" form.

About 3% of the ingested material is excreted in the urine within 4 hours, the bulk is "conjugated."

Traces of "free" and "conjugated" phenol are excreted in the feces. Faint traces of "free" phenol are eliminated in the exhaled air.

5. The fate of a sublethal or an approximately lethal dose of phenol, at the end of the first 4 hours after its oral administration to the rat, is as follows:

Roughly 60% (46–82%) of the amount administered is destroyed. Destruction is most rapid, per unit of time, after sublethal doses (0.2 g./kg.).

Roughly 40% (18–53%) of the ingested material is still present in the carcass at the end of the 4 hours. Following a dose of 0.2 g./kg. most of this is present as "conjugated" phenol; after doses of 0.4 and 0.6 g./kg. the larger proportion is present as "free" phenol.

Traces of "free" and "conjugated" phenol are excreted with urine and feces.

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Effect of Nicotinic Acid Intake on the Coenzyme 1 Content of Chick Tissues [1]

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Introduction

Recently Briggs, *et al.*, showed that nicotinic acid is an essential dietary component for the chick (1). Employing a synthetic ration, they were able to use the chick as an experimental animal in a biological assay for nicotinic acid. It has been known for some time that nicotinic acid is a part of coenzyme 1, which is intimately concerned in tissue metabolism. Therefore, it seemed to be of interest to investigate the coenzyme 1 and nicotinic acid content of tissues of the chick to determine what quantitative change they undergo with various levels of nicotinic acid intake. Similar studies have been made with the dog and the pig (2, 3, 4, 5), and with the blood and muscle of pellagrins (6, 7, 8, 9, 10). These experiments seem to indicate, in general, that a nicotinic acid deficiency results in a lowered coenzyme 1 value in the muscle and the liver, but not in the other tissues studied. Conflicting reports have been published regarding blood coenzyme 1 levels in clinical pellagra, but the more recent experiments have shown that while blood coenzyme 1 may be increased slightly in normal persons by an excess dietary intake of nicotinic acid, there is no significant decrease in the coenzyme 1 content

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins; to The Wilson Laboratories, Chicago, for solubilized liver (liver Fraction L); to Wilson and Company, Inc., Chicago, for gelatin; and to Allied Mills, Inc., Peoria, Illinois, for soy bean oil.

We are grateful to G. M. Briggs, Jr., and T. D. Luckey for the care and feeding of the chicks used in these experiments.

[2] Wisconsin Alumni Research Foundation Fellow.

of the blood of pellagrins. In the muscle of persons suffering from pellagra, however, there seems to be some decrease in coenzyme 1, related to the severity of the symptoms. Axelrod, Madden, and Elvehjem (4), and von Euler, *et al.* (11) have reported that there is no increase in the coenzyme 1 content of the tissues of rats fed excess nicotinic acid, and Dann and Kohn (12) report only a slight increase. This is in agreement with the fact that rats do not require a dietary source of nicotinic acid.

Methods

Details of the methods and diets used in the care of the chicks have been reported in a previous paper (13). The basal diet still contained a small amount of nicotinic acid, but it was low enough so that definite differences in growth between the chicks on the basal diet and those on the nicotinic acid supplemented diet could be noted at the end of four weeks. As before, a supplement of 1.5 mg. nicotinic acid per 100 g. ration seemed to be sufficient for maximum growth, so that the 10 mg. per 100 g. ration supplement was far in excess of the requirement.

The coenzyme 1 was determined by the yeast fermentation method of von Euler (14) and Myrbäck (15) as later modified by Axelrod and Elvehjem (16). In addition, more effective methods were employed in preparing the tissues for analysis to prevent the destruction of the coenzyme 1 by nucleotidases known to be present. All operations between killing of the chick and the weighing of the tissue were performed in a cold room at a temperature of 10°C. The animal was decapitated and the head dropped immediately into liquid air. Afterwards the brain was removed. The other tissues to be analyzed (liver, heart, breast, and leg muscle) were quickly removed from the animal and also frozen immediately in liquid air. After freezing, the tissues were stored between slabs of dry ice. The frozen tissue was then pulverized in a steel crusher.[3] Approximately 1 g. samples of the powdered tissue were weighed on a torsion balance, then washed into 5–8 cc. of boiling water. The extracts were boiled 2–3 minutes, then allowed to cool, and made up to a volume of 10 cc. Appropriate aliquots of the extracts were taken for analysis, in order that there should be between 5–25 μg. of coenzyme 1 present in each flask. These values represent the limits of the standardization curve. Analyses were made in a Barcroft respirometer at 30°C. In addition to the substances mentioned by Axelrod and Elvehjem, 0.1 mg. muscle adenylic acid and 20μg. cocarboxylase per flask were needed to give maximum carbon dioxide evolution with this particular sample of brewers' yeast (17). The maximum fermentation values, which were usually obtained at 20 minutes, were compared with a standard curve. The standard used in these determinations was a sample of cozymase obtained from von Euler's laboratory. It was shown to have a maximum potency of 80% according to a nicotinic acid assay and a spectrographic analysis. This standard was also checked by three methods against a sample of

[3] This apparatus was designed by G. A. LePage and V. R. Potter of McArdle Memorial Institute.

coenzyme 1 from Schlenk's laboratory—yeast fermentation, nicotinic acid assay, and spectrographic analysis. The same relative purity was observed in all three cases thus showing that these methods of calibration give equally accurate results.

Nicotinic acid was determined in liver, breast and leg muscle, by the microbiological method of Snell and Wright (18) using the modification of Krehl, Strong, and Elvehjem (19).

Discussion

The results of these experiments are presented in Table I. It is evident that in the case of breast and leg muscle there is a definite and direct relationship between the nicotinic acid and coenzyme 1 content of the tissues and the dietary nicotinic acid. On the basal diet the values were appreciably lower than on the diet supplemented with 1.5 mg. per 100 g. ration. The latter level of supplement, in addition to the small amount of nicotinic acid in the basal diet, is sufficient for optimum growth, as may be observed from the weights of the chicks at four weeks given in the table. There is no increase in the weight of the chicks when the supplement is increased to 10 mg. per 100 g. ration, but there is a marked increase in nicotinic acid and coenzyme 1 content of breast and leg muscle.

In the liver, no such definite correlation is possible. In the two lower levels the coenzyme 1 and nicotinic acid values are relatively the same. The coenzyme 1 content of the 10 mg. group shows a slight increase which may or may not be significant. The tissue nicotinic acid at this level, however, is significantly higher. This is not in agreement with data on other species. The dog and pig both show decreases in coenzyme 1 in the liver, as well as the muscle, on low nicotinic acid diets. This may be essentially a species difference in coenzyme 1 storing capacity of the liver, or it may be merely a difference in experimental conditions. The dogs and pigs used in other experiments were adult animals, while the chicks used here were rapidly growing young animals.

There does not appear to be a significant increase in coenzyme 1 content of heart tissue corresponding to the increase in dietary nicotinic acid. This is in accordance with observations on other species; also heart tissue may be expected to remain constant in composition even under rigorous conditions because of its importance to the animal as a whole.

Brain tissue is low in coenzyme 1 in all nicotinic acid levels, but there seems to be a slight increase at the 10 mg. level.

A definite advantage in making simultaneous determinations of nico-

tinic acid on three of the tissues studied was that a comparison could be made between the nicotinic acid values and coenzyme 1 values, not only

TABLE I

Coenzyme 1 and Nicotinic Acid Content of Tissues of the Chick on a Varying Dietary Intake of Nicotinic Acid

No.	Wt. at 4 wks. (*g*)	Breast muscle			Leg muscle			Liver			Heart *Co. 1 (*Obs.*)	Brain *Co. 1 (*Obs.*)
		*Co. 1 (*Obs.*)	†Total N. A. (*Obs.*)	‡N. A. bound as Co. 1	*Co. 1 (*Obs.*)	†Total N. A. (*Obs.*)	‡N. A. bound as Co. 1.	*Co. 1 (*Obs.*)	†Total N. A. (*Obs.*)	‡N. A. bound as Co. 1		
No nicotinic acid supplement												
1801	185	50	15.1	9.2	95	35.2	17.6	490	117	91	185	
1802	215	150	22.8	27.8				540	116	100	280	
1804	185	115	19.1	21.3	<10	20.5	<2	515	144	95	180	49
1805	180	90	18.8	16.7				180	90	33	210	<10
1806	200	70	14.4	12.9	75	17.6	13.9	610	121	113	320	95
1.5 mg. nicotinic acid per 100 g. ration												
1819	270	160	10.9	29.7				530	137	98	155	40
1820	290	130	21.4	24.1	150	46.9	27.8	555	100	103	330	
1821	290	120	38.2	22.2	175	53.4	32.4	190	114	35	130	80
1822	260	215	34.5	39.9	200	35.4	37.0	415	113	77	210	60
1823	190	280	59.1	51.9				510	89	94	270	100
10 mg. nicotinic acid per 100 g. ration												
1825	260	325	165	60	230	46	43	740	171	137	265	200
1826	275	850	153	157				565	210	104	290	97
1828	285	665	185	123	340	86.3	63	610	179	113	260	55
1829	245	510	181	94				670	325	124	170	105
1830	240	685	162	127	285	71	53	965	178	178	305	175

Nicotinic acid and coenzyme 1 analyses are expressed in μg. per g. fresh tissue. The Co.1 values represent an average obtained from 3 or more analyses.

* "Co.1 (*Obs.*)" refers to analyses of coenzyme 1 by the yeast fermentation method.

† "Total N. A. (*Obs.*)" refers to analyses of nicotinic acid by the microbiological method.

‡ "N. A. bound as Co.1" is calculated from "Co.1 observed" by dividing by the factor 5.4.

to check the coenzyme 1 analysis, but to determine the nicotinic acid which was either free in the tissues or bound in some other form. In Table I, the column which has been headed "nicotinic acid bound as Co. 1" is a representation of this comparison. In order to obtain these figures, the coenzyme 1 values were divided by 5.4, the ratio of the molecular weight of coenzyme 1 to the molecular weight of nicotinic acid. Therefore, the total nicotinic acid values should exceed the calculated values for nicotinic acid bound as coenzyme 1. Within experimental error, except for one value (Chick No. 1819, breast muscle), this is actually the case.

It is interesting to note that in breast muscle and liver there seems to be a rough correlation between the nicotinic acid, either present as such or bound in forms other than Co. 1, and the nicotinic acid of the diet. On the basal diet, the "free" nicotinic acid of breast muscle amounted to an average of only 9% of the total nicotinic acid. In animals receiving the 1.5 mg. and 10 mg. supplements, the "free" nicotinic acid was 45% and 50% of the total, respectively. The livers of animals receiving the basal diet and those receiving the 1.5 mg. per cent supplemented diet showed "free" nicotinic acid values of approximately 35%; at the highest level of dietary nicotinic acid this figure rose to 60%. This seems to indicate that although in the breast muscle of a deficient animal, the nicotinic acid not bound as Co. 1 is greatly depleted, the liver still retains a considerable store of the free vitamin. It is only upon increasing the supplement far above the level necessary for optimum growth that the "free" nicotinic acid content of the liver is increased. This was not observed by Dann and Handler (2) in their work on normal and black-tongue dogs. Their "unbound" nicotinic acid, which excluded both coenzymes 1 and 2, remained relatively constant in a given tissue regardless of the nicotinic acid content of the diet—about 40% in liver and 20% in muscle.

An interesting comparison may be made between the coenzyme 1 values and the per cent of "free" nicotinic acid at a given dietary level of nicotinic acid. While the coenzyme 1 content of breast muscle increased greatly at the highest level of dietary nicotinic acid, the per cent of "free" nicotinic acid remained the same as in the group receiving the 1.5 mg. supplement. The increase in "free" nicotinic acid in breast muscle then corresponded roughly to the growth response of the chicks to the various supplements. In liver, however, the "free" nicotinic acid was the same for the basal and 1.5 mg. supplemented groups, and

doubled when the supplement was increased to 10 mg. per cent, while the coenzyme 1 values remained approximately the same.

Summary

1. A direct correlation was observed between the tissue nicotinic acid and coenzyme 1 levels in muscle and the nicotinic acid of the diet. A similar correlation was noted between dietary and tissue nicotinic acid in the liver, but the coenzyme 1 levels increased only slightly in the group on the highest nicotinic acid supplement.

2. Heart and brain showed little if any increase in coenzyme 1 content with increase in dietary nicotinic acid.

3. A comparison is made between the nicotinic acid of the tissues as determined by the microbiological method, and the nicotinic acid as calculated from the coenzyme 1 content of the tissues.

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Purified Prothrombin and Thrombin: Stabilization of Aqueous Solutions

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Introduction

In blood or plasma thrombin can exist for only a few minutes because plasma contains antithrombin in relatively high concentration (1). If thrombin is purified in such a manner as to remove all antithrombin, aqueous solutions are then found to be stable for approximately two days at room temperature (2). Thereafter activity begins to disappear quite rapidly because of inherent instability of the protein. To prevent such loss in activity, Milstone (3) has used glycerol to good advantage.

It has now been found that a large number of commonly available carbohydrates will also prevent loss of activity for many days and even months. The glycosides appear to be particularly effective, and that is especially to be expected when the glycoside is quite soluble. It can also be shown that glycerol is most effective in the region of 75% concentration.

Experimental

Materials and Methods

Beef prothrombin and thrombin were prepared and assayed by methods already described (4, 5, 6).

It was at once apparent that an accelerated stabilization test was needed, and the following procedure was adopted: Neutral thrombin or prothrombin solutions were placed in test tubes, assayed, and adjusted to a convenient concentration range of 1000 to 1500 units per cc. The tubes were then sealed and placed in an oven set at 50°C. The activity remaining at the end of 48 hours was then recorded in per cent of original activity remaining. The test is quite severe, and moderately effective compounds are not discovered. The latter are of relatively little interest, but in those instances where they are it is necessary to make tests at room temperature over protracted periods of time.

Comparative Loss of Activity at Room Temperature and at 50°C. for 48 Hours

In order to interpret results obtained at 50°C. it was necessary to calibrate the test against common experience at room temperature. The frequent use of glycerol in enzyme work and its proven effectiveness with thrombin prompted its selection for these comparative tests. At room temperature (Fig. 1) full activity is maintained for approximately a month provided the glycerol concentration is near 50 per cent. When the concentration is less than 25 per cent there is very little stabilization.

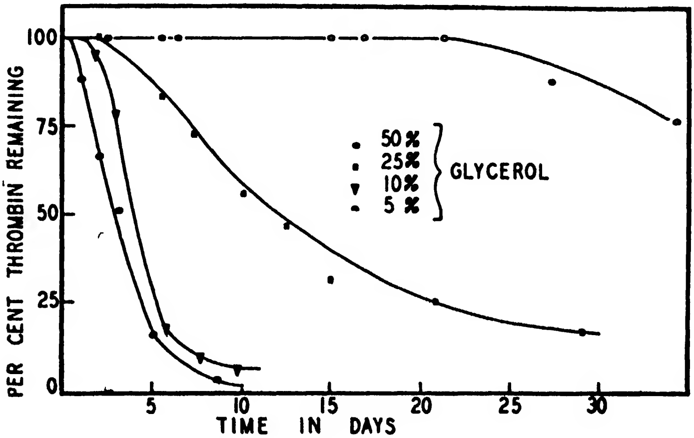

FIG. 1

Effect of Glycerol Concentration on Thrombin Activity at Room Temperature

In fact it is so meager that it cannot be detected with the accelerated test. More extensive work at 50°C. gave the following results:

Per Cent glycerol	25	50	75	80	90
Activity remaining	0	18	65	30	19

For best stabilization it is not sufficient to add glycerol in progressively greater amounts, because a definite optimum is reached near 75 per cent concentration. This observation complicates the task of formulating a theory which will explain the action of glycerol, and also leads one to inquire whether other enzymes respond in a similar manner.

Stabilization with Carbohydrates

Saturated solutions of the carbohydrates and their derivatives vary considerably in their effectiveness. The following are some compounds

which show definite influence when studied at room temperature: *l*-rhamnose, α-methyl-*l*-rhamnoside, *l*-arabinose, *l*-mannose, sodium gluconate, lactose, β-lactose-octaacetate, maltose, inositol, sorbitol, and mannitol.

The common disaccharide, sucrose, is the most interesting compound studied to date (Table I). It gives progressively greater protection as the concentration increases. At 66% concentration over 95% of the thrombin remains unchanged. It has thus more influence than glycerol at its optimum concentration. The hydrolytic products fructose and

TABLE I

Per Cent Activity Remaining After Heating at 50°C. for 48 Hours

Compound (Saturated Solution)	Thrombin	Prothrombin
Water	0	0
Glucose	10	
α-Methyl-*d*-glucoside	60	0
β-Methyl-*d*-glucoside	80	0
d-Xylose	0	
β-Methyl-*d*-xyloside	17	0
β-Methyl-*l*-xyloside	22	0
α-Methylxyloside	12	
d-Galactose	4	
α-Methyl-*d*-galactopyranoside	17	1
β-Methyl-*d*-galactopyranoside	78	
β-Methyl-*d*-galactofuranoside	70	
60% Sucrose	79	
66% Sucrose	97	12
75% Glycerol	65	0
d-fructose	46	
Ethyl-lactoside	28	

glucose give relatively low stabilization values. In the case of glucose only 10% of the thrombin activity remained.

Monomethylation of the glucose molecule increases the stabilizing properties more than six times. It is not known whether the differences between α-methyl-*d*-glucoside and β-methyl-*d*-glucoside are statistically significant.

Protective properties of xylose can only be demonstrated at room temperatures, and while the xylosides show much improvement over xylose itself they are not much more valuable than glucose.

Galactose is of interest because α-methyl-*d*-galactoside and β-methyl-

d-galactoside exhibit quite different properties. The respective figures being 17 and 78. Certainly the difference is significant from the standpoint of the data, but the meaning remains obscure and tempts speculation. Another derivative, β-ethyl-*d*-galactofuranoside, was found to be equivalent to β-methyl-*d*-galactopyranoside. The 1:4 configuration gives excellent results, and it is interesting to recall that sucrose is a glycoside which contains a furan ring structure in the fructose portion of the molecule.

Stabilization of Prothrombin Solutions

Preliminary work with this proenzyme shows that circumstances are quite different from what might be predicted from data on thrombin (Table I). Glycerol appears to be of no special value, and sucrose solutions contained only 12% of the original activity at the end of the 48 hour test period. If ones conception of a proenzyme requires that the molecule is quite similar to the active enzyme itself, these negative results do not easily fall into such a pattern.

Discussion

It has not been possible to deduce a rigid rule which will serve to predict whether a given sugar will protect thrombin from losing activity. The glycosides are generally active, but exceptions to that rule have also been found. The factor of carbohydrate concentration is an important phase of this new problem which has not been studied, because it is only one of many variables that are significant.

Apparently the inherent instability of the protein thrombin resides in those portions of the molecule which are easily damaged by heat. If that assumption is accepted the subject of thrombin stability is definitely related to the broader topic (7) of alterations in proteins by heat, and may ultimately be correlated with the problem of resistance to digestive enzymes as illustrated, for example, by the case liver proteins (8). Certainly the ease with which thrombin activity can be measured makes thrombin a useful tool for the study of this subject.

I wish to thank Dr. L. A. Sweet, Dr. W. L. Evans, and Dr. R. M. Hann for placing small quantities of many carbohydrates at my disposal.

Summary

Saturated solutions of a large number of carbohydrates and their derivatives have the property of preserving the activity of thrombin.

The glycosides appear to be especially effective. Glycerol shows an optimum range near 75% concentration.

Prothrombin solutions are not stabilized as readily as thrombin solutions.

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The Mechanism of Color Production in Escherichia Coli Cultures Containing Sulfonamides

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Introduction

The formation of yellow, orange, or red colors in a synthetic medium during *in vitro* testing of the bacteriostatic activity of various sulfonamides against *Escherichia coli* has been noted in this laboratory. This phenomenon was observed after 20–36 hours' incubation at 37°C. and appeared *first* in those concentrations immediately below the bacteriostatic range of the compounds where growth equalled or exceeded that of the controls. On continued incubation (48–72 hours) colors became visible in the presence of greater and lesser sulfonamide concentrations than that concentration first showing color. After about 72 hours' incubation, it was found that the color intensity had become proportional to the sulfonamide concentration provided that the bacterial growth had approached that of the controls. Color formation was most noticeable in those cultures which contained sulfonamides of relatively low activity and was not observed in tests in which sulfanilamide or more active compounds were used.

Experimental

The synthetic medium which was employed throughout this investigation was composed of the following:

Na_2SO_4	5.0 g.
NH_4NO_3	5.0 g.
$MgSO_4$	0.2 g.
K_2HPO_4	4.0 g.
Asparagine	3.0 g.
Glucose	10.0 g.
Distilled Water to	800.0 ml.

The medium was adjusted to pH 7.4 with $N/5$ NaOH and filtered through paper. Tubes containing 4.0 ml. amounts of medium were

autoclaved at 12 lbs. for 10 minutes. To each tube was added 1.0 ml. of a dilution in distilled water of the sodium salt of the sulfonamide being studied. The inoculum consisted of 0.1 ml. of a 1:10,000 dilution of a 24-hour culture of *E. coli* strain No. 2017.

The conditions occurring in cultures growing in the absence of sulfonamides were investigated with the aim of discovering the contributing factors responsible for the production of color. It was found that after 20–24 hours' incubation, the pH of the cultures had fallen to approximately 4.5, and a considerable amount of nitrite had been formed. It was apparent, therefore, that the conditions were satisfactory for the diazotization of any primary aromatic amine capable of such a reaction under these circumstances. Since most sulfonamides contain an unsubstituted amino group joined to the benzene ring, diazotization of such compounds under the conditions existing in *E. coli* cultures should be possible.

To test this deduction, a solution was prepared similar to the culture medium outlined above, with the exception that the ammonium nitrate was replaced with sodium nitrite and the pH was adjusted to 4.6 with 3 *N* acetic acid. To this uninoculated solution were added various sulfonamides which had given color in previous bacteriostatic tests with *E. coli*; each was adjusted to a final concentration of 0.001 *M*. After standing at room temperature for 15 minutes, color appeared in all the solutions. These colors were red, orange, or yellow depending upon the sulfonamide employed and were qualitatively similar to those which had been produced in the *E. coli* cultures.

The above experiment was repeated under still simpler conditions where a solution was prepared to contain only 0.5% sodium nitrite adjusted to pH 4.6 with acetic acid. In this experiment the sulfonamides listed in Table I were employed. It will be noted that of the 21 compounds tested, each of 15 contained a free *p*-amino radical while 6 were derivatives lacking this group. *p*-Aminobenzoic acid was also included. It can be seen from Table I that each of the compounds having a free *p*-amino group produced color under these simple conditions. None of the others displayed this property.

From this series of experiments, it appears that sulfonamide drugs are capable of diazotization under the conditions existing in *E. coli* cultures. However, for actual color formation the resultant diazonium salts must couple with another molecule. Under the simplified conditions of the last experiment, the only compounds available for coupling were the

undiazotized molecules of the sulfonamides themselves. It seems, therefore, that the rate of the diazotization is slower than the rate of the coupling reaction under these conditions and that the individual compounds may undergo diazotization and simultaneous condensation with the undiazotized molecules.

TABLE I

Compounds according to Color Formation[1]

Yellow		
*Sulfadiazine		
*Sulfamethazine		
*Sulfamerazine		
p-Aminobenzenesulfonyl-glycine		
Sulfanilamide		
p-Aminobenzoic acid		
Orange to Red		
2-*p*-aminobenzenesulfonamido-5-carboxy-thiazole		
*2-"	"	4-methyl-5-carboxy-thiazole
2-"	"	4-carboxymethyl-5-carboxy-thiazole
*2-"	"	4′-carboxy-sulfanilide
2-"	"	2′-carboxy-sulfanilide
2-"	"	benzoic acid
4-"	"	" "
2-"	"	4-carboxy-thiazole
2-"	"	4-methyl-5-carboxymethyl-thiazole
*Sulfathiazole		
No color		
2-benzenesulfonamido-4-carboxy-thiazole		
2-	"	4-methyl-5-carboxy-thiazole
N^4-phthalyl-sulfanilamide		
N^4-phthalyl-sulfathiazole		
N^4-succinyl-sulfanilamide		
N^4-succinyl-sulfathiazole		

* pptn. occurred—solution takes place on addition of alkali.
[1] In general, colors may be intensified on addition of alkali.

This explanation of the color-producing reaction was further supported by finding that it was not dependent on the presence of sulfonamide during the growth of the culture. When the sulfonamides mentioned in Table I were added in 0.001 *M* concentration to 24-hour cultures of *E. coli*, colors appeared within a few minutes which were qualitatively similar to those produced in the simple acid-nitrite solution. Under

these conditions similar colors were obtained with sulfanilamide, sulfathiazole, sulfadiazine and sulfamerazine, although these sulfonamides had not given color in tests where *E. coli* was grown in the presence of the compounds. When 0.0001 *M* PABA[1] was added to culture medium containing these drugs in 0.001 *M* concentration, characteristic color formation was observed after inoculation with *E. coli* and incubation for 48 hours. The principal action of PABA in this case was merely that of inhibiting these highly active sulfonamides. This inhibition permitted growth and nitrite production to occur in the presence of sufficient sulfonamide to produce visible quantities of the colored product. The additional color contributed by the diazotization of the PABA in the concentration present was negligible.

p-Aminobenzenesulfonyl-glycine was selected for more detailed study. This drug was added to fully grown *E. coli* cultures and to acid-nitrite solution in 0.001 *M* concentration. Although the colors produced in both instances appeared to be similar in quality, the color in the culture was slightly intensified over that in the acid-nitrite solution. Spectrophotometric measurements (using the Beckman Quartz Spectrophotometer Model DU) indicated that the culture contained at least one colored component in addition to that found in the acid-nitrite solution. Because of the recent work of Spink and Vivino (5) and Mayer (4) the possible rôle of PABA was further investigated. It was found that PABA in biological concentrations (0.01–10.00 γ per 5 ml.) produced no observable color when added to either grown culture or to acid-nitrite solution. Likewise, no additional color was formed when similar concentrations of PABA were added to acid-nitrite solutions already containing 0.001 *M* sulfonamide. It would appear, therefore, that although PABA in 0.001 *M* concentrations can be diazotized and coupled to produce color, the relatively small amounts naturally present in *E. coli* cultures (2, 3) cannot explain the additional color produced. It was further observed that when the nitrite in a fully grown *E. coli* culture was destroyed by ammonium sulfamate and *N*-(1-naphthyl)-ethylenediamine hydrochloride was added, as in the test of Bratton and Marshall (1), a yellow color was produced. A similar reaction took place when either *p*-aminobenzenesulfonyl-glycine or PABA was used in place of the Bratton and Marshall coupling agent. This indicates that there exists in the medium in which *E. coli* has grown one or more substances already in a diazotized form, but which require the presence of a coupling agent

[1] PABA = *p*-Aminobenzoic acid.

for the production of color. The nature of this additional component is being investigated further.

Discussion

From the experiments described above, it seems clear that the production of color in *E. coli* cultures containing sulfonamides is dependent on the production of nitrite and acid in the presence of a concentration of sulfonamide adequate to produce a detectable amount of azo dye. These conditions result in the diazotization of a portion of the sulfonamide and its subsequent coupling with the undiazotized molecules. It has also been demonstrated that other primary aromatic amines such as PABA, when added in similar concentrations, may take part in reactions of this type. In a complex system such as that produced by the metabolism of bacteria, small quantities of PABA and of other primary aromatic amines may also be present. It is probable therefore, that these amines when diazotized, will couple with themselves, or with the sulfonamide present, and produce small quantities of colored substances different from the major product, *i.e.*, diazotized and coupled sulfonamide. The colors produced by these side reactions are, to a large extent, masked by the intensity of the color resulting from the major reaction, and can best be demonstrated by spectrophotometric measurements. Although PABA, when present in 0.001 *M* concentration, was shown (Table I) to be able to diazotize and couple with itself (and presumably with sulfonamides), concentrations of PABA, even greater than those reported by Landy, *et al.* (2, 3), were not sufficient to give visible color. Thus, it seems likely that PABA is not involved in the major reaction resulting in the color production observed in *E. coli* cultures grown in the presence of sulfonamide. However, PABA can act as a sulfonamide inhibitor, permitting the organisms to produce nitrite and acid in the presence of quantities of sulfonamide adequate for the production of visible color.

Summary

Color production was observed in *E. coli* cultures growing in a synthetic medium in the presence of sub-inhibitory quantities of sulfonamides of low bacteriostatic activity. This color could also be elicited with highly active sulfonamides, providing growth was allowed to take place by the addition of PABA. Color could similarly be produced by adding sulfonamides to fully grown cultures and solutions of acidified sodium nitrite. It is suggested that these colors result from diazotization of the sulfonamide and subsequent coupling with itself in the pre-

sence of nitrite and acid produced by the organisms. Inter-reactions between sulfonamide and other diazotized substances synthesized by the organisms were detected, but these were slight compared to the chief reaction. Color production was observed only with those compounds possessing a free *p*-amino group on the benzene ring.

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Determination of Pyridoxin and Pseudopyridoxin*

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Introduction

Methods for the determination of pyridoxin have been based on the growth of rats (1), yeast (2), and bacteria (3), and also on the development of color with chemical reagents (4). The discovery of pseudopyridoxin (5) at once raised the question of whether this form of the vitamin is active toward higher animals, and whether it is measured by the above assay methods. It now appears very probable that pseudopyridoxin is not active in the rat (6). Therefore, if biological materials are to be assayed for their pyridoxin value, and if further studies on pseudopyridoxin are to be carried out, methods for the determination of each form separately must be available.

In the present work the usefulness of several microbiological methods for pyridoxin has been investigated. Methods for determining pseudopyridoxin have also been studied, and some preliminary observations regarding its chemical nature have been made.

Methods

Preparation of Samples. Except where otherwise indicated, samples were prepared for assay by suspending a weighed amount (approximately 1 g.) in 50 cc. of 0.1 *N* HCl and autoclaving for 15 minutes at 15 pounds pressure. Such suspensions were used directly for the *Neurospora* assays. For the *L. casei* and yeast assays the suspension was adjusted to pH 4.5 with 2.5 *N* sodium acetate solution, diluted to 100 cc., and filtered through quantitative paper (*e.g.* No. 40 Whatman). An aliquot of the clear filtrate was adjusted to pH 6.8 and made to a convenient volume for assay. The final solution contained about 0.05 μg. of pyridoxin per cc. for the *Neurospora* and *L. casei* assays and about 0.01 μg. per cc. for the yeast assay.

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Neurospora sitophila method

The test organism was the "pyridoxin-less" mutant of *N. sitophila*[1] (7). It was carried on yeast extract-glucose-agar slants prepared by dissolving 0.5 g. of glucose, 1 g. of yeast extract (Difco), and 1.5 g. of agar in 100 cc. of water. These cultures were incubated at 30°C. for 72 to 96 hours, and then stored in the refrigerator until needed. Transfers were made at monthly intervals, and at each monthly transfer several extra stock cultures were prepared, and were used to grow inoculum as required during the month.

To grow inoculum, a loop of conidia was transferred to an agar slant and incubated 72 to 96 hours at 30°C. The conidia from such a recently grown culture were then used as inoculum by placing them at one end of the assay tubes. These tubes were 1.5 cm. in diameter, 40 cm. long, open at each end, and segments of about 5 cm. at the ends were bent upward at 45° angles.

The composition of the basal medium used was as follows.[2]

Sucrose	15 g.
Agar	20 g.
Ammonium tartrate	5 g.
KH_2PO_4	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
NaCl	0.1 g.
$CaCl_2 \cdot 2H_2O$	0.1 g.
$FeCl_3$	200 μg.
$ZnSO_4$	150 μg.
$CuCl_2$	40 μg.
$MnSO_4 \cdot 4H_2O$	20 μg.
Ammonium heptamolybdate	20 μg.
Boric acid	10 μg.
Biotin (free acid)	4 μg.
Water to make	1000 cc.

The washed agar used in this medium was prepared by extracting 200 g. of finely ground agar with 6 liters of water stirred continuously for 4 hours. The agar was allowed to settle, and the supernatant liquid poured off. It was then transferred to a cheesecloth and as much water as possible removed by wringing. This process was repeated 6 times, and then repeated three times more with alcohol in place of water. The product was finally dried at 35° C.

The biotin used was a sample of the methyl ester[3] which was hydrolyzed by autoclaving in 0.1 N HCl, neutralized, and diluted to 0.1 μg. per cc. The sucrose was Heyden No. 3, obtained from the American Refining Company.

To set up an assay, the various ingredients of the basal medium were dissolved in a small amount of hot water, and the solution diluted to four-fifths the final

[1] The culture of *N. sitophila* used in this work was very kindly furnished by Dr. G. W. Beadle.

[2] Suggested by Dr. Beadle in a personal communication.

[3] Obtained from the S.M.A. Corp., Chagrin Falls, Ohio.

volume. A 20 cc. portion was pipetted into a glass stoppered graduate cylinder, an aliquot of the standard solution[4] or extract of the sample added, and the volume adjusted to 25 cc. Duplicate 10 cc. aliquots were pipetted into the assay tubes, which were then plugged with cotton, sterilized at 15 pounds pressure for 15 minutes, cooled, and inoculated. A metal rack was used to hold the tubes in a horizontal position with the bent ends pointing upward, so that as the medium cooled it solidified in the form of a continuous layer through the length of the tube. The inoculated tubes were incubated at 30° C., and the progression of the frontier of mycelia noted at 12 hour intervals over a period of 72 hours.

Lactobacillus casei Method

The procedure of Landy and Dicken (3) was followed with certain modifications. These included the addition of 1 μg. of *p*-aminobenzoic acid per 10 cc. of the basal medium, the use of a casein hydrolyzate prepared as for the nicotinic acid assay (8), and the use of inoculum grown in the riboflavin basal medium (9) plus 1 μg. of riboflavin per 10 cc.

Since large inocula lead to high blanks and modify the response of *L. casei* to pyridoxin (10), the cell suspension to be used as inoculum was extensively diluted. Cells from a 24 hour, 10 cc. culture were centrifuged, suspended in 10 cc. of 0.9 per cent saline, centrifuged again, and resuspended in 4 cc. of saline. In the earlier part of the work 1 cc. of the latter suspension was diluted with 85 cc. of saline as recommended by Landy and Dicken (3). Later a sufficient amount was added to 85 cc. of saline to produce a barely visible turbidity (usually 5 to 10 drops). In either case one drop of the final suspension was used to inoculate each assay tube.

Yeast Method

This procedure was carried out as described by Atkin, *et al.* (2) except that the cultures were grown in 50 cc. Erlenmeyer flasks. The flasks were attached to a rack which was lowered into a 30° C. water bath, and the entire set was mechanically shaken approximately 60 times per minute throughout the growth period (16–18 hours).

Results

Neurospora Assay

A typical set of growth curves is given in Fig. 1. Levels above 0.04 μg. of pyridoxin per 25 cc. of medium gave only a slight increase in the rate of growth. Since tubes containing no added pyridoxin were able to support a small amount of growth, the medium apparently was not completely free from pyridoxin. It will be noted that the rate of growth decreased noticeably toward the end of the incubation period, and that in general the rate was not linear with time.

[4] Synthetic pyridoxin hydrochloride was used as the primary standard throughout this work. The results have been expressed directly as pyridoxin, without calculation to the free base.

In order to investigate the effect of variations in the size of inoculum, a series of tubes was prepared with 0.02 μg. of pyridoxin in each, and duplicate tubes were given light, medium, and heavy inoculations respectively. The rate of growth in the various tubes did not differ markedly; if anything the rate in the lightly inoculated ones was slightly faster than in the others.

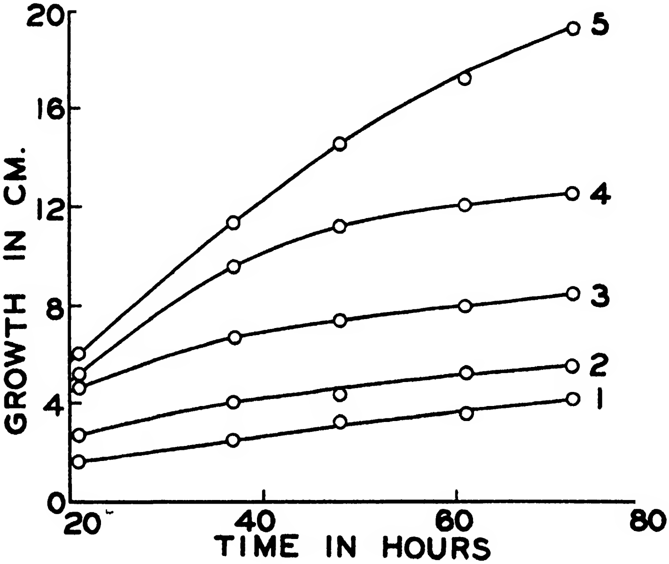

FIG. 1

Response of *Neurospora sitophila* to Pyridoxin

1. 0.00 μg. of pyridoxin per 25 cc. of medium
2. 0.01 μg. of pyridoxin per 25 cc. of medium
3. 0.02 μg. of pyridoxin per 25 cc. of medium
4. 0.03 μg. of pyridoxin per 25 cc. of medium
5. 0.04 μg. of pyridoxin per 25 cc. of medium

The mycelium produced when suboptimal amounts of pyridoxin were present was very light, and did not appear to penetrate the agar. It thus seemed likely that the amount of growth depended upon the amount of the vitamin near the surface of the agar, rather than on the total quantity in the tube. Two sets of tubes in which each tube contained 15 and 10 cc. of medium (0.03 and 0.02 μg. of pyridoxin) respectively were prepared and inoculated. The rate of growth in the two sets was the same. Consequently, differences in the growth rates in duplicate tubes would be

expected if the physical character of the solidified agar in various tubes was different.

The usefulness of the assay as a measure of the pyridoxin content of natural materials was tested by applying it to several grain samples and comparing the results with values obtained on the same samples by the yeast and rat assay methods. Since in our hands the growth rate was variable, it seemed preferable to base the assay on the extent of growth after some convenient interval. Two standard curves of this sort are given in Fig. 2, and the complete data for one assay are collected in

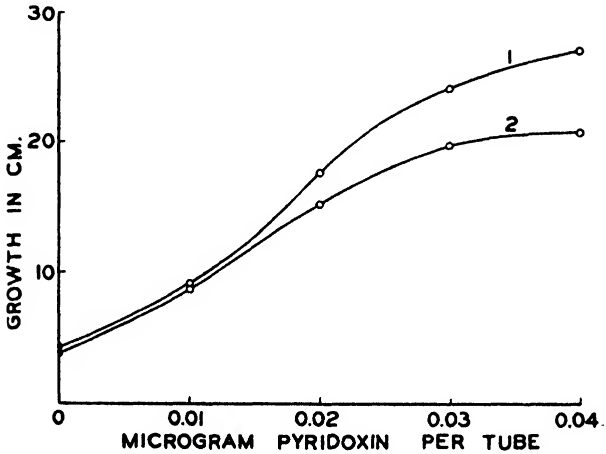

FIG. 2

Standard Curves for Pyridoxin Determination with *Neurospora sitophila*

1. 72 hours incubation.
2. 60 hours incubation.

Table I. The pyridoxin content of the samples listed in Table I was calculated by interpolation on a 72 hour growth curve. Results obtained in this manner are compared with yeast and rat assay[5] values in Table II.

L. casei Assay

A typical response of *L. casei* to graded amounts of pyridoxin is illustrated in Fig. 3. This standard curve is similar to that reported by Landy and Dicken (3), except that only about one-half as much pyridoxin was needed to reach a titration of 7–8 cc. as they reported to be

[5] These rat assay values were made available through the courtesy of C. A. Elvehjem.

necessary. The theoretical maximum titration of slightly over 10 cc. was never reached unless the tubes contained 2–3 μg. of pyridoxin.

TABLE I

Sample Calculation for Neurospora Assay

Sample	Amount added per tube*	Growth after 72 hours	Pyridoxin per tube	content per gram of sample
	mg.	*cm.*	*micrograms*	
Whole Wheat	10	10.2	0.009	0.9
	10	8.7	0.007	0.7
	20	22.3	0.026	1.3
	20	19.8	0.022	1.1
Whole oats, No. 1	10	13.2	0.013	1.3
	10	20.3	0.023	2.3
	20	25.5	0.032	1.6
	20	26.3	—	—
Whole oats, No. 2	10	11.8	0.011	1.1
	10	13.5	0.014	1.4
	20	22.3	0.026	1.3
	20	24.0	0.030	1.5

* Added as a suspension (see text).

TABLE II

Comparison of the Neurospora, Yeast, and Rat Assay Methods for Determination of Pyridoxin

Sample	Neurospora	Yeast	Rat
		micrograms per gram	
Whole wheat, no. 1	1.5	4.1	4.6
	1.0		
	6.2*		
Whole wheat, no. 2	8.2	3.3	4.7
Whole wheat, no. 3	6.2	3.8	4.8
Whole oats, no. 1	1.7	1.4	1.7
	1.5		
Whole oats, no. 2	1.4	2.2	2.7
	1.8		

* No explanation can be offered for the lack of agreement in this series of assays.

The fact that the lowest level of pyridoxin, 0.05 μg. in Fig. 3, produced a titration of 6 cc. suggested that even lower levels should be included in the standard series. However, subsequent experiments showed nearly as high titration at the first level of the series even though only 0.0125

μg. of pyridoxin was used. Below this level the "jump" in titration did not occur.

Throughout these experiments it was observed that the bacterial response was very slow. Visible growth did not appear during the first 48 hours of incubation in culture tubes containing 0.2 μg. or less of pyridoxin. Repeated 24 hour subculturing on the modified Landy and Dicken basal medium plus 2 μg. of pyridoxin per tube resulted in progressively poorer growth, until after 3 transfers practically no growth occurred. Further-

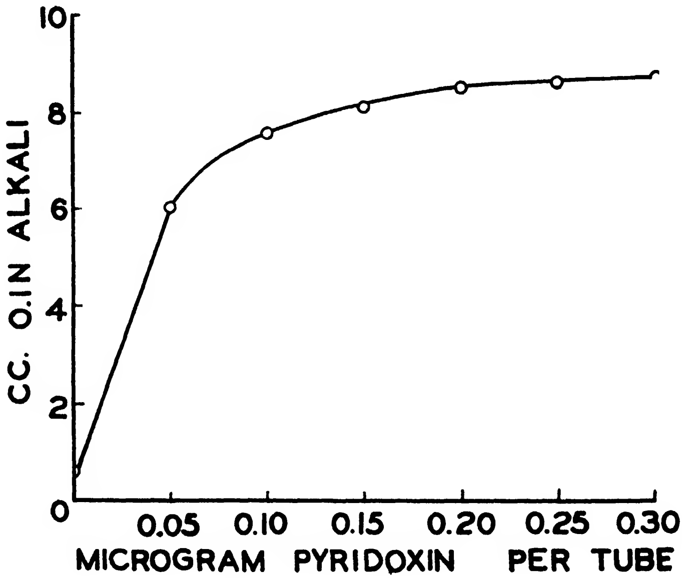

FIG. 3
Standard Curve for Pyridoxin Determination with *L. casei*

more duplicate assay tubes often gave widely divergent titration values, and in fact the entire standard curve varied considerably from assay to assay. On some occasions no growth resulted in any of the tubes of the standard series, while on other occasions all the tubes showed approximately the same amount of growth.

In spite of these difficulties an effort was made to apply the method to the determination of the pyridoxin content of four natural materials, *viz.* viobin (defatted wheat germ), ground whole oats, yeast, and a distillers residue sample. The values obtained were 20, 5, 1100, and 10 μg. per g.

respectively, whereas 12, 2.7, 115, and 5.2 μg. per g. respectively were found on the same samples by the rat assay method[5] (1). A mixture containing known amounts of synthetic thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxin was also assayed. In this case the value found amounted to 91 per cent of the pyridoxin known to be present.

Modifications of the Basal Medium

Attempts to improve the modified basal medium by doubling the concentration of each component separately or by doubling the concentration of the whole medium were unsuccessful. The inclusion of $(NH_4)_2SO_4$, additional sodium acetate, and glutamic acid in the medium likewise failed to increase the response of the organism. Alanine in amounts of 25 to 100 μg. per tube caused a slight stimulation of growth at low levels of pyridoxin.

An effort was also made to prepare supplements to the medium from natural materials known to promote growth of *L. casei*. Various procedures for removing the pyridoxin originally present were tried. These included adsorption on fuller's earth, English fuller's earth, and active charcoal from aqueous solutions at various pH levels, both before and after autoclaving the material, continuous extraction with butanol, photolysis (11), and oxidation with $KMnO_4$. One or more of these procedures were applied to yeast extract, whole autolyzed yeast[6], peptone, solubilized liver[7], and vitab[8]. In no case was a supplement obtained which materially improved the bacterial response without at the same time raising the blank titration to excessively high levels.

Pseudopyridoxin

In connection with the above work, the effect of various chemical agents on pure pyridoxin was also investigated. In particular it seemed likely that oxidative treatment would destroy the vitamin and offer a means for removing it from natural materials. However, the surprising observation was made that H_2O_2 treatment of synthetic pyridoxin re-

[6] Obtained from the Difco Laboratories, Detroit.

[7] The solubilized liver was obtained from the Wilson Laboratories, Chicago. It is that portion of an aqueous liver extract precipitated by 70 per cent ethanol, and subsequently rendered water soluble by enzyme digestion.

[8] Vitab is a rice bran concentrate, obtainable from the National Oil Products Company, Harrison, N. J.

sulted in a product which was actually several fold more active toward *L. casei* than pyridoxin itself (Table III). In this experiment 5 μg. of pyridoxin was dissolved in 40 cc. of water, 10 cc. of 3 per cent H_2O_2 added, and the solution allowed to stand 4 hours at room temperature. The excess H_2O_2 was then removed with MnO_2, the solution filtered, and made to volume for assay.

Shortly after this preliminary experiment was completed, the paper of Snell, *et al.* (5) appeared announcing the existence of a substance similar to pyridoxin which showed enhanced activity toward lactic acid bacteria, and was called pseudopyridoxin. It seems probable that the same substance was involved in the present work. It has, therefore, been referred

TABLE III

Effect of Various Chemical Agents on Pyridoxin

Treatment	Pyridoxin Before	Pyridoxin After	Recovery
	μg. per cc.		*per cent*
H_2O*	0.050	0.050	100
0.1 *N* Hydrochloric acid*	0.050	0.054	108
1.0 *N* Hydrochloric acid*	0.050	0.044	88
0.1 *N* Sodium hydroxide*	0.050	0.125	250
1.0 *N* Sodium hydroxide*	0.050	0.125	250
Hydrogen peroxide	0.050	0.30	600
Cystine	2.5	5.62	225
Potassium permanganate	2.5	0	0
Cyanogen bromide	0.050	0.092	184

* 5 micrograms of pyridoxin in a volume of 50 cc., autoclaved 15 minutes at 15 lbs. pressure, neutralized and made to a volume of 100 cc.

to by the same name, and the amounts of it present in various materials have been expressed in terms of pyridoxin as was done by Snell, *et al.* (5). The absolute values obtained cannot, of course, be compared directly because different test organisms were employed.

Yeast assay of H_2O_2-treated solutions failed to show enhanced activity (6). Apparently this organism measures only unchanged pyridoxin.

However, *L. casei* appears to respond to both forms. This was established by testing pyridoxin solutions which had not been autoclaved. Sufficient tubes for two standard curves were set up in the usual manner, except that the standard solutions were not put in, until after the tubes were autoclaved. One standard solution contained 0.1 μg. of pyridoxin hydrochloride per cc. while the other was the H_2O_2-treated solution

(Table III) diluted to a concentration equivalent to 0.025 μg. of the original pyridoxin per cc. Each of these solutions was sterilized by filtration through a Seitz funnel before use. The response of the organism is shown in Table IV.

It is evident that *L. casei* utilizes both pyridoxin and pseudopyridoxin. Measurement of the latter with this organism, therefore, can be accomplished only by means of a differential analysis in conjunction with some method for determining pyridoxin alone. We have used the yeast method for this purpose. We have not yet been able to secure usable results with *S. lactis*, which is stated not to require unchanged pyridoxin (12) and which, therefore, should offer a means of determining the pseudo form directly. The maximum growth obtained with this organism in our hands has been too slight to permit satisfactory turbidity measurements with the Evelyn colorimeter, and a more sensitive turbidimeter was not available.

TABLE IV

Response of Lactobacillus casei to Unheated Pyridoxin

Tube No.	Pyridoxin* Amount added	Pyridoxin* Titration 0.1 N alkali	Treated pyridoxin† Amount added	Treated pyridoxin† Titration 0.1 N alkali
	μg. per tube	*cc.*	*μg per tube*	*cc.*
1	0.00	1.1	0.00	1.2
2	0.05	2.3	0.0125	3.4
3	0.10	2.6	0.025	4.7
4	0.15	2.9	0.0375	5.8
5	0.20	3.2	0.050	5.7
6	0.25	4.9	0.0625	6.4
7	0.30	4.9	0.075	6.8
8	0.5	5.2	0.125	6.7

* Sterilized by filtering through Seitz filter.

† Hydrogen peroxide-treated pyridoxin sterilized by filtering through Seitz filter.

Some indication of the reliability of this differential analysis was provided by repeated assay of the same solution of H_2O_2-treated pyridoxin. The values found in six successive assays of one preparation were 15, 14, 9, 12, 15, and 13 μg. of pseudopyridoxin per cc. Although considerable variations were observed the results, nevertheless, are sufficiently concordant to show that a several-fold increase in activity does result from the treatment of pyridoxin solutions with H_2O_2.

Several other methods of treating pyridoxin were found to give solutions having increased activity toward *L. casei*. These results are collected in Table III. The cystine treatment consisted in autoclaving a solution of 250 μg. of pyridoxin, 50 mg. of cystine hydrochloride, and 250 mg. of sodium acetate in 50 cc. of water for 1 hr. at 15 pounds pressure. The pH of the solution was 7.2. The $KMnO_4$ oxidation was carried out by treating a solution of 250 μg. of pyridoxin in 50 cc. of water with 0.1 cc. of 4 per cent $KMnO_4$ solution for 1 hr. at room temperature. The cyanogen bromide treatment was carried out according to the procedure of Swaminathan (13).

TABLE V

Formation of Pseudopyridoxin by Light and Aeration

Treatment	pH	Pyridoxin Before	Pyridoxin After	Pyridoxin Loss	Pseudo-pyridoxin formed
		μg. per cc.	*μg. per cc.*	*per cent*	*μg. per cc.*
Artificial light*	6.8	25	9.6	62	none
Artificial light†	13	5.0	0.6	88	3.5
Natural light*	6.8	25	19	24	none
Aeration†	13	1.0	0.26	74	5.0

* 24 hour irradiation. For artificial light the solutions were exposed in an open beaker 8 inches below a 300 watt bulb. For natural irradiation the solutions were exposed to bright diffused daylight.

† 36 hour irradiation with artificial light and simultaneous aeration by bubbling air through the solution.

In view of the recent note of Hochberg, *et al.* (11) the effect of light on pyridoxin and pseudopyridoxin solutions was also investigated. The results are shown in Table V. In this case the pseudopyridoxin formed was determined by the differential method described above. It is evident that very extensive destruction of pyridoxin occurred, and that pseudopyridoxin was simultaneously produced when the irradiation was carried out in an alkaline medium.

However, in neutral solution, pseudopyridoxin is destroyed by light. This was determined by exposing a neutral solution of 250 μg. of H_2O_2-treated pyridoxin in 100 cc. of water to the light from a 300 watt bulb at a distance of 8 inches for 36 hours. The pseudopyridoxin content of the solution dropped from 16 to 0.5 μg. per cc. At the same time the pyridoxin content was reduced from 2.5 to 0.3 μg. per cc.

Yeast Assay

In general, the yeast growth method for pyridoxin appeared to give very satisfactory results. As pointed out above, this method apparently does not measure the pseudo form, and the results obtained agreed very favorably with rat assay values on the same samples (Table II). The wheat samples listed in Table II were prepared for the yeast assay by extracting with 0.44 *N* H_2SO_4 according to Atkin, *et al.* (2), while the oat samples were extracted by autoclaving with 100 cc. of 0.1 *N* H_2SO_4 for 1 hr. at 20 pounds pressure.

TABLE VI

Activity of Pyridoxin Derivatives for Yeast

Compound	Activity†
I. 2-Methyl-3-hydroxy-4,5-bis(hydroxymethyl)-pyridine (Pyridoxin)	1.0
Derivatives of 2-Methyl-pyridine*	
II. 3-Hydroxy-4,5-bis-(acetoxymethyl)-	1.0
III. 3-Acetoxy-4,5-bis-(acetoxymethyl)-	0.05
IV. 3-Hydroxy-4,5-bis-(bromomethyl)-	0.70
V. 3-Hydroxy-4-methoxymethyl-5-hydroxymethyl	0
VI. 3-Hydroxy-4,5-dimethyl-	0
VII. 3-Ethoxy-4,5-bis-(hydroxymethyl)-	0
VIII. 3-Hydroxy-4-methyl-5-hydroxymethyl-	0.03
IX. 3-Hydroxy-4,5-bis-(aminomethyl)-	0
X. 3-Ethoxy-4,5-bis-(aminomethyl)-	0
XI. 3-Hydroxy-4,5-epoxydimethyl-	0

* Obtained through the courtesy of Dr. Karl Folkers, Rahway, New Jersey.

† Calculated on molar basis.

The availability of a series of pyridoxin derivatives for the yeast used in this assay was also determined. From the data in Table VI it appears that only the diacetate and the bis-bromomethyl derivative are active for this organism. These results in general are similar to the activities of these substances for *L. casei* and rats (10).

Discussion

The variable response of the organism, the non-linearity of the standard curve, the low maximum titrations, the failure of the organism to grow when subcultured on the basal medium plus excess pyridoxin, and the high apparent pyridoxin values[9] found on natural products, all indi-

[9] These high values can hardly be attributed to stimulation by fatty materials in view of the manner in which the samples were prepared for assay; cf. Carpenter and Strong, 14.

cate that the Landy and Dicken basal medium for *L. casei* (3) is deficient in some unknown growth factor or factors. When this information is coupled with the fact that *L. casei* also responds to pseudopyridoxin, which is not active in the animal (rat), it becomes obvious that the Landy and Dicken assay method is of no value as a means of determining the pyridoxin content of biological materials.

As carried out in the present work, the *Neurospora* method was subject to several disadvantages. One troublesome feature was the shape of the frontier of mycelium. This frequently was not well defined, especially at the lower levels of pyridoxin. On the contrary the frontier was sometimes wedge shaped, diagonal, or a few strands grew a centimeter or more beyond the main body of mycelium. In such cases the measurement of growth was necessarily somewhat arbitrary.

Furthermore the growth responses were not always as regular as the data presented would indicate. In about a quarter of the assays carried out, individual tubes were encountered in which the growth rate did not seem to be directly dependent on the amount of pyridoxin present. This variation was so marked in one case that two growth curves such as those in Fig. 1 actually crossed.

In spite of these difficulties the method appears capable of yielding useable results, and could doubtless be further improved. It provides an entirely independent check on other methods for determining pyridoxin, and gives values in the same order of magnitude as found by animal and yeast assay.

In view of the deficiency of the *L. casei* medium used in this work, it is obvious that pseudopyridoxin determinations carried out on natural materials with the help of *L. casei* are necessarily subject to considerable uncertainty. For this reason the pseudopyridoxin work was mainly confined to preparations obtained from synthetic pyridoxin.

Of the various methods for forming pseudopyridoxin which have been reported to date, the most effective are oxidative procedures. These include treatment with H_2O_2, autoclaving with a large excess of cystine, and aeration of alkaline solutions. We are inclined to believe, therefore, that the active substance is an oxidation product of pyridoxin. Whether or not the substance concerned in the present study is identical with the pseudopyridoxin of Snell, *et al.* (5, 12) cannot be determined definitely at the present time, but seems probable in view of the similar results obtained by the cystine treatment.

To date no pseudopyridoxin preparations free from unchanged pyridoxin have been secured, although a number of attempts to separate the

two substances were carried out. These included adsorption on norite, fractional elution with water, alcohol, and ammonia solutions, and attempts to destroy pyridoxin preferentially by photolysis.

Summary

The utility of *Neurospora sitophila* as an assay organism for pyridoxin was investigated. The method gives values of the same order of magnitude as those determined by rat assay, but is subject to considerable variations and needs further study before it can be recommended for routine use.

The *L. casei* assay for pyridoxin suggested by Landy and Dicken was thoroughly studied. On the best available basal medium, the test organism responds both to pyridoxin and pseudopyridoxin, and probably also to other unknown nutrilities. It, therefore, cannot be regarded as a reliable measure of the pyridoxin value of biological materials.

Pyridoxin was converted to pseudopyridoxin in several different ways, and preliminary observations regarding the chemical nature and properties of the latter were made.

Direct comparison of the yeast and rat methods for determining pyridoxin was carried out, and good agreement was found. The availability of a series of pyridoxin derivatives for the strain of yeast used in this assay was studied. The yeast method appears to be the best microbiological assay procedure for pyridoxin available at the present time.

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Growth, Reproduction, and Lactation in Rats Maintained Through Four Generations on Highly Purified Diets[1]

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Introduction

In the past few years conflicting results have been reported concerning the nutritional requirements of rats for reproduction and lactation. Although it is generally agreed that a diet adequate for growth does not necessarily have to be adequate for reproduction and lactation, opinions vary among investigators as to what constitutes a good highly purified ration that will enable an animal to pass through one or more normal cycles of growth, reproduction, and lactation. Several groups of workers have claimed moderate or good success with diets containing the established B complex vitamins in crystalline form. With such a diet Jukes (1) obtained some success on reproduction in rats although the growth rate was below normal. Unna (2), Unna and his co-workers (3), Henderson, *et al.* (4), and Folley, *et al.* (5) have been able to maintain rats on synthetic diets through at least three generations. However, lactation, for the most part, was poor, and average weaning weights of the surviving litters were low. Richardson, *et al.* (6) used a diet which they found to be excellent for growth and adequate for reproduction and lactation. In a subsequent communication (7), however, they stated that a supplement of liver extract was required for successful lactation. The importance of *p*-aminobenzoic acid and to a lesser extent of inositol was emphasized by Sure (8), who found that normal lactation was only obtained when these supplements were added to the diet. Similar conclusions were reached by Climenko and McChesney (9), except that their results indicated that inositol was the more important lactation factor.

[1] A preliminary report of this investigation has appeared (18).

In this laboratory two strains of rats have been kept on highly purified diets through four generations. The growth of these animals was excellent in every generation. Of particular interest was the observation that the mothers invariably lost considerable weight during the nursing period.

Experimental

The rats used in these experiments were of two kinds, belonging to the black or piebald Evans-Long strain, and to the Wistar strain.

Several diets were employed (Table I). Of those listed, only one (diet R-3) proved to be totally inadequate in supporting lactation. Animals on this ration suffered frequently from diarrhea. Of the other diets, those that contained a combination of lard and Crisco (diets R-4 and R-5) gave slightly better results in so far as average weaning weights of litters were concerned. Diet R-5 containing 15% fat was most frequently used in these experiments. Evans, *et al.* (10) and Maynard and Rasmussen (11) have shown that lactation improved when the fat content of the diet was increased.

TABLE I

Composition of Diets

Component	Diet *R-1*	*R-2*	*R-3*	*R-4*	*R-5*	*R-6*
Purified Casein (Labco or Smaco)	30	30	30	30	30	30
Salts (Osborne and Mendel)	5	5	5	5	5	5
Ruffex[1a]	2	2	2	2	2	2
Lard	10	—	—	2	5	—
Crisco	—	8	—	8	10	10
Corn oil	—	2	10	—	—	—
Sucrose	53	53	53	53	48	53

Supplements were the same for all diets. They were per kilogram: thiamine, 20 mg.; riboflavin, 20 mg.; pyridoxin, 20 mg.; calcium pantothenate, 40 mg.; choline chloride, 500 mg.; α-tocopherol, 20 mg.; vitamin A and D concentrate, 40 mg.

[1a] α-Cellulose preparation obtained from Eimer and Amend, New York.

In our early experiments the protein level of the diet was 20%. This ration was the same as diet R-1 except for the lowered protein content and resembled closely the diet employed by Richardson, *et al.* (6). Wistar rats, placed on this diet when 21 days old, grew at a normal rate, but complete failure met attempts to raise litters upon mating 20 females. Upon increasing the protein content to 30%, successful lactation was

obtained. These results are in agreement with those of Daggs and Tamboulian (12), and of Sure (13).

Four generations were raised on the highly purified diets. Table II presents the data on reproduction and lactation for both strains of rats used. It can be seen that the number of young weaned is significantly smaller for both strains of rats on the purified rations than the number weaned on the control diet of Purina dog chow. However, the growth of these animals was excellent throughout the four generations.

TABLE II

Data on Reproduction and Lactation

Strain	Generation	No. of Females	Litters born	Litters weaned	Young weaned	Litter size	Average weaning weight g.
Wistar	Parent	27	25	15	101	6.7	38.3
"	F_1	35	26	15	79	5.3	33.8
"	F_2	13	12	4	16	4.0	34.2
"	F_3	7	4	1	4	4.0	29.6
"	Total	82	67	35	200	—	—
"	Controls on Purina dog chow	15	14	11	79	7.0	34.5
Evans and Long	Parent	4	4	4	33	8.3	26.4
"	F_1	15	14	11	68	6.0	26.5
"	F_2	15	12	4	24	6.0	25.8
"	Total	34	30	19	125	—	—
"	Controls on Purina dog chow	10	10	8	75	9.4	25.3

Figs. 1 and 2 show typical growth curves of litters raised on the purified diets and on Purina dog chow. The Evans-Long strain rats grew at a rate equal to that of litter mates on the control diet. Also, the slopes of the growth curves for the parent and second filial generations are about the same. Similar results were observed with the Wistar rats.

A very important observation has been made during lactation. Invariably the mother lost considerable weight, particularly during the latter part of lactation. Immediately after separating the rat from the weaned litter, she regained sharply the weight lost. Fig. 3 shows the fluctuations in weight of a Wistar rat (F_1 generation) through a

period covering three successful pregnancies. It will be observed that soon after mating the sharp rise in the curve is due to pregnancy, while the precipitous drop corresponds to parturition. Then, in every case

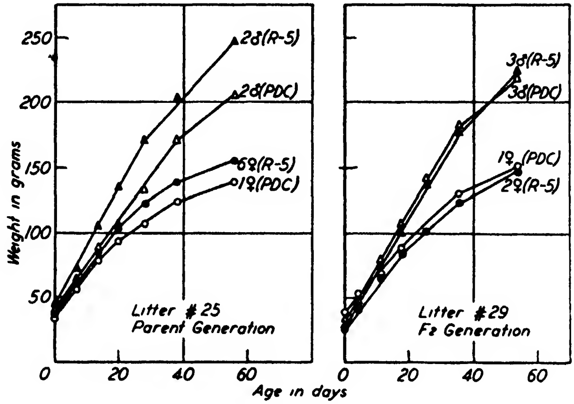

FIG. 1

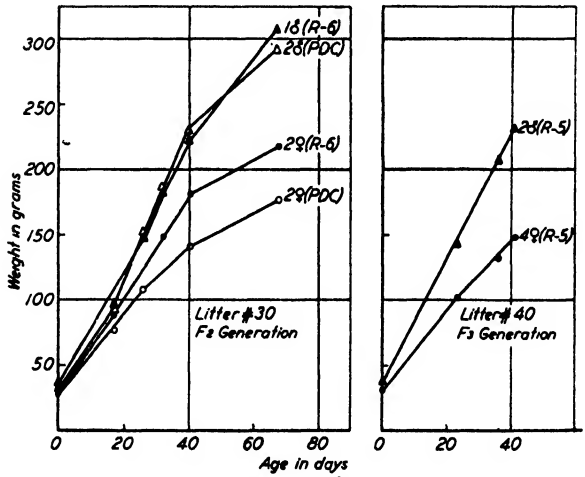

FIG. 2

there is a slight gain in weight during the first few days of lactation with a subsequent sharp drop for the remainder of the lactation period. Upon separating the mother from the young, the curve rises sharply denoting a gain in weight again. This cycle is repeated in the same manner for the other two pregnancies.

Under the conditions of our experiments the average loss in weight for Wistar rats during lactation was 40 g. In some cases the loss was much larger, reaching 70 g. This enormous loss occurred when a mother was taking care of a large litter consisting of eight or more young. For a 250 g. rat, such a drop in weight amounts to almost 30% of the body weight. A similar phenomenon was observed in the case of the Evans-Long rats except that the loss in weight during lactation was not quite as great. This difference may be due to the fact that an adult female of the Evans-Long strain weighs less than an adult Wistar female.

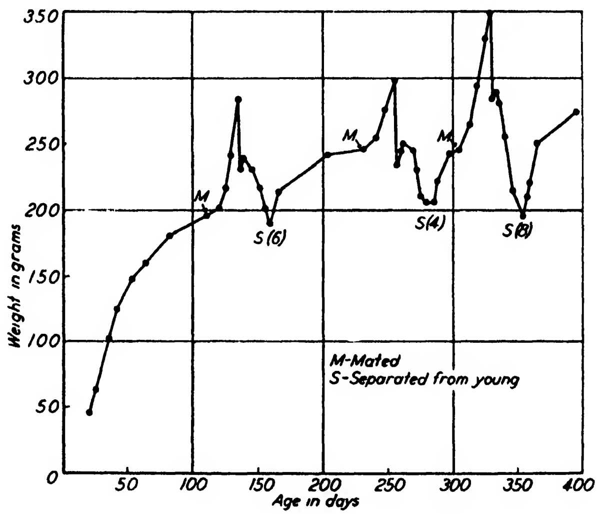

FIG. 3

Some other observations made on lactating rats kept on highly purified diets are worth recording. It was noticed that several Evans-Long rats ate their feces. Also, there was a frequent collapse of the mother in the late stages of lactation (15th–18th day). In such cases, either the litter was destroyed, or the mother died from complete exhaustion. These rats appeared very shabby and emaciated. Due to the constant nursing of their young, several mothers developed ulcerated mammary glands.

It was concluded from these observations that the diet was inadequate in supplying some constituent needed for maximum milk production. To rule out the possibility that one of the primary foods might have

been ingested in insufficient quantities, the following supplements were tested by supplying them *ad libitum* to lactating mothers: casein, sucrose, and lard. None of these improved the lactation performance of the rats. In fact, the mothers fed the lard supplement destroyed their young during lactation. These rats ate the lard ravenously, eating as much as 8 g. per day. Apparently the consumption of such large amounts of lard interfered with the ingestion of other food constituents.

Other supplements that were tried without any beneficial effect in preventing the high weight loss of lactating mothers were: Biotin, 0.1

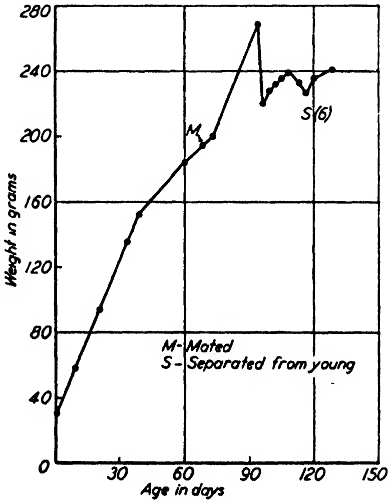

FIG. 4

μg./day, *p*-aminobenzoic acid plus inositol, each 0.5 mg./day, and yeast nucleic acid, 30 mg./day.

Brewers' yeast[2] when fed to rats as a supplement (500 mg./day) to the purified ration prevented the loss in weight of lactating mothers.[3] (Fig. 4).

[2] Brewers' yeast powder, strain K. We are indebted to Anheuser-Busch, Inc., St. Louis, Mo., for a generous supply of this material.

[3] This loss in weight of lactating mothers was also prevented in the case of mice kept on highly purified diets, by a supplement of a folic acid concentrate (19). Preliminary experiments on rats, using this concentrate have shown an improved average weaning weight of litters and only a slight loss in the weight of lactating mothers.

Discussion

The use of several highly purified diets in which the content and type of fat differed was planned because of certain results that were obtained with mice in this laboratory. When Crisco was the sole source of fat in the diet, mice failed to reproduce. A suspicion that the essential fatty acids were present in insufficient quantity in this fat arose when several cases of resorption occurred. On addition of either lard or corn oil to the diet, resorptions disappeared and successful lactation was obtained. These two fats are known to be rich sources of linoleic acid. Apparently, Rogers, *et al.* (14), in raising mice through two generations on an artificial diet, encountered a similar deficiency in essential fatty acids since in addition to Crisco, their ration contained a supplement of linoleic acid. However, in the past year we have found that it is possible to raise litters on diets containing Crisco as the only fat (diet R-6). Whether there has been a change in the preparation of Crisco or the early work with mice was inconclusive has not been determined.

Throughout the four generations the rats on the purified diets compared favorably with control animals in every respect except during lactation. Here we have observed the sharp loss in weight of the mothers. These rats exert themselves to the utmost in supplying nourishment to their young. Since the diet apparently fails to supply some factor in sufficient quantity to satisfy the demands of lactation, the mother in an effort to continue maximum milk production breaks down her own body tissue to supply the missing substance necessary for adequate lactation. This idea is substantiated by the observation that rats nursing small litters lost less weight than mothers taking care of large litters. When the tissue breakdown reaches a critical stage, the mother either succumbs or the litter is destroyed. The several cases of coprophagy observed in the case of the black rats are highly significant since they point to a nutritional deficiency while under the strain of lactation.

The idea of the existence of a specific lactation factor, a substance that initiates the lactation mechanism and without which no milk can be produced by the mother, has been put forward by Mapson (15), and by Nakahara, *et al.* (16, 17). The former was unable to obtain successful lactation in rats on a purified diet containing yeast as a source of the vitamin B complex. Approximately 70% of the young born died within 3 days after parturition. Upon feeding whole liver or an aqueous extract of it, normal lactation was obtained. Mapson gave the name "physin"

to the substance in liver responsible for this effect. Nakahara also assumes the existence of specific lactation factors, and in a series of papers has attempted to demonstrate the necessity of two such substances.

The results of our experiments do not lend support to the idea of a true lactation factor since it was possible to wean a moderately high percentage of litters through several generations on highly purified rations. What they indicate, however, is that a lactagogue, some substance causing increased milk production, is lacking in the purified diets. Such a substance seems to be present in yeast.

Summary

1. Several highly purified rations have been employed in raising rats belonging to two different strains through four generations.

2. Growth on these diets was excellent throughout the four generations and compared favorably with that of controls kept on Purina dog chow.

3. Invariably, the mothers lost weight during lactation which was regained as soon as they were separated from the young.

4. Of the several supplements used to prevent the loss in weight during lactation only brewers' yeast was found to be beneficial.

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Nature of Carbon Monoxide Inhibition of Biological Nitrogen Fixation*

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Introduction

Small quantities of CO in the atmosphere inhibit fixation of molecular nitrogen in both the symbiotic system represented by inoculated red clover plants (1) and the asymbiotic system of free-living *Azotobacter* (2, 3). For interpreting this result in terms of enzyme mechanism it is desirable to determine the nature of the inhibition—whether competitive or non-competitive with respect to N_2 gas. A preliminary trial with red clover (1) suggested non-competitive inhibition, but confirmation of this finding with *Azotobacter* is desirable. In the relatively long-time plant experiments secondary effects arising from the primary inhibition may greatly influence the observations. It is not surprising then that the rates of fixation in replicate samples vary markedly, a circumstance which might mask certain characteristics of the inhibition. Moreover, the experiments with plants require two to three months for completion in contrast to the single day necessary for an equivalent micro-respiration trial using *Azotobacter*. It became evident early in the investigation that a large number of tests would be essential for decision on some aspects of the problem; these could be most readily completed in the short-time experiments with *Azotobacter*.

Theory

Usually the experimenter bases his conclusions as to the nature of an inhibition by noting whether the activity of the inhibitor depends only on its own concentration or also on the concentration of substrate.

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The specific question is: does the *relative velocity constant* at a given concentration of inhibitor depend on the concentration of substrate? Although answering this question may suffice for qualitative decision, methods based on consideration of mechanism of reaction may be required for unequivocal conclusions. Such methods possess several advantages. First, they are ordinarily more sensitive, as all results are considered simultaneously rather than by piecemeal comparisons. Second, they furnish definite criteria for recognition of different types of competition so that one or more of these may be detected when occurring together. Finally, they provide estimates of important constants of the reaction, a procedure which is facilitated by use of regression statistics.

The most convenient forms of the equations for testing the type of inhibition are those proposed by Lineweaver and Burk (4). The following considerations are based on their treatment together with supplementary information which Dr. Burk has recently furnished us in a private communication. Detailed derivation of the equations for Types I and II has been furnished by Wilson (5); those for the other types involve similar algebraic manipulations.

Type I. Reaction in absence of inhibitor

If the following schema represents essentially the enzyme reaction which occurs

$$E + S \rightleftarrows ES \tag{1}$$
$$ES \rightarrow E + P \tag{2}$$

E = concentration of enzyme
ES = concentration of enzyme-substrate complex
S = concentration of substrate
P = measurable product of reaction

velocity equation 3 can be derived:

$$v = \frac{V(S)}{K_s + (S)} \tag{3}$$

In this equation, v represents the velocity at substrate concentration (S); V the maximum velocity when enzyme is saturated with substrate, and K_s equilibrium constant of reaction 1. A more useful form is obtained by taking the reciprocal of both sides since this results in a linear equation (4).

$$\frac{1}{v} = \frac{1}{V} + \left(\frac{K_s}{V}\right)\frac{1}{(S)} \tag{4}$$

Type II. Competitive inhibition

In addition to reactions 1 and 2, the inhibitor combines with E at the same point in effect as does the substrate so that it competes with the latter; hence inhibition will depend on both concentration of inhibitor and of substrate.

(5) $E + I \rightleftarrows EI$ (inactive with dissociation constant, K_i).

The velocity equation in this case is

$$\frac{1}{v_i} = \frac{1}{V^*} + \left[1 + \frac{(I)}{K_i}\right]\left(\frac{K_s}{(S)}\right)\frac{1}{(S)} \tag{6}$$

Where v_i is velocity at substrate concentration (S) and V^* (equal to V) is maximum velocity, both in presence of inhibitor.

From equations 4 and 6 the following alternative form can be derived

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i}\frac{(I)}{K_s + (S)} \tag{7}$$

Type III. Non-competitive inhibition

In addition to reactions 1, 2, and 5, a further reaction occurs

(8) $ES + I \rightleftarrows ESI$ (inactive with dissociation constant, $K_{esi} = K_i$).

That is, the inhibitor combines with enzyme independent of the presence of substrate and hence attacks E and ES with equal affinity at a point not attacked by substrate.[1]

The velocity equations are:

$$\frac{1}{v_i} = \left[1 + \frac{(I)}{K_i}\right]\left[\frac{1}{V^*} + \left(\frac{K_s}{V^*}\right)\frac{1}{(S)}\right] \tag{12}$$

$$\frac{v}{v_i} = 1 + \frac{(I)}{K_i} \tag{13}$$

V^* in this case equals $V(1 + I/K_i)$.

Dr. Dean Burk has recently referred us to two other types of inhibition in which the percentage inhibition also depends on concentration of substrate but which differ from the strictly competitive type. These have not been previously recognized, but they certainly should be considered in interpretation of kinetic data. We designate them with the names he has used in our correspondence.

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[1] Alternatively, leading to same velocity equations, the inhibitor may attack some component, A, with which ES reacts to form P.

(9) $ES + A \rightarrow P$

(10) $A + I \rightleftarrows AI$ (with dissociation constant, K_{ai})

In this case the affinity of the inhibitor for the enzyme is zero

(11) $(1/K_i = 0)$

and the observed dissociation constant which we call K_i is actually K_{ai}.

Type IV. "Uncompetitive" inhibition

The inhibitor combines with ES but not with E so that the kinetic equation is

$$\frac{1}{v_i} = \left[1 + \frac{(I)}{K_i}\right]\left(\frac{1}{V^*}\right) + \left(\frac{K_s}{V^*}\right)\left(\frac{1}{(S)}\right) \tag{14}$$

An example of this "enzyme-complex inhibition," which is the converse of competitive inhibition, is the combination of azide with the *oxidized* form of the Atmungsferment but not with its reduced form (6).

Type V. "Quadratic" inhibition

If, as in many biological processes, the reaction is not a simple one capable of formulation by the Michaelis-Menten schema, a more complex one such as the following may represent the true course of events:

$$E + S \rightleftarrows ES \xrightarrow{\text{slow}} P \xrightarrow{\text{fast}} B \longrightarrow C \tag{15}$$

$$C + I \rightleftarrows CI \tag{16}$$

C represents some compound which is eventually involved in the reaction in order to make ES available. The kinetic equation in this case is a quadratic so that the slopes change as will be discussed later, but corresponding to equation 13:

$$\frac{C}{C_{total}} = \frac{K_i}{K_i + (I)} \tag{17}$$

See Winzler (6), Commoner (7), and Lineweaver and Burk (4, Fig. 6).

To decide the type of inhibition, one first determines as accurately as possible the velocity constants of the reaction *over as wide a range of substrate concentration as is practical* and at two or more concentrations of inhibitor. If the reciprocal of the velocity constant, $1/v$, is plotted against the reciprocal of the concentration of substrate, $1/(S)$, straight lines should result with the following characteristics:

Type I. In the absence of inhibitor, a straight line results whose slope/intercept equals K_s, the dissociation constant of the enzyme-substrate complex.

Type II. In strictly competitive inhibition, the intercept remains constant, but the slope is increased by $(1 + (I)/K_i)$. The apparent K_s = slope/intercept increases therefore by the same factor.

Type III. In strictly non-competitive inhibition, both slope and intercept are increased by the same quantity, *viz.*, $(1 + (I)/K_i)$ so that K_s remains constant.[2]

[2] In previous papers from this laboratory which dealt almost exclusively with competitive inhibition (5, 8, 9), the statement was made that the slopes should be equal in non-competitive inhibition. In a private communication discussing our single paper which was concerned with this type of inhibition (1) Dr. Dean Burk kindly pointed out that the slopes as well as the intercepts must increase in strictly non-competitive inhibition. In the subsequent correspondence he drew to my attention the possibility that we might be dealing with an inhibition exemplified by Types IV and V and made certain suggestions with regard to experimental test of these possibilities. We are indebted to Dr. Burk for his valuable suggestions and advice which aided us greatly in formulating the theoretical background and interpreting the experimental results of this research (P. W. W.).

Type IV. The slope may change by a factor less than the intercept indicating that the inhibitor has a greater affinity for ES than for E. In the limiting case I combines only with ES, and the slope does not change, but the intercept increases by $(1 + (I)/K_i)$. The apparent K_s decreases.

Type V. The lines obtained for different values of the inhibitor have different intercepts and begin with near zero slopes, then change to a common slope equal to that obtained when (I) is zero (see Lineweaver and Burk, 4, Fig. 6).

Another convenient method for distinguishing between strictly competitive and strictly non-competitive inhibition is based on equations 7 and 13; v/v_i is plotted against (I) for two or more values of (S). If the inhibition is competitive, straight lines are formed with unit intercept but different slopes; if non-competitive, the lines coincide since Equation 13 does not contain (S) as a factor.

Experimental

Application of the methods described in the foregoing section will be illustrated by a detailed consideration of experiments undertaken to determine the mechanism of inhibition of biological nitrogen fixation by carbon monoxide.

Methods

The velocity of the nitrogen fixation reaction was measured by k, the specific rate constant. It was determined in the Warburg microrespirometer (*Azotobacter vinelandii*) or by periodic harvest of red clover plants. Details of the methods are given in our earlier publications (1, 2, 3). Any special technic adopted will be indicated in the text.

Red Clover Experiments

Our initial studies (1) were made with the symbiotic nitrogen fixation system of inoculated red clover plants. Although the data suggested non-competitive inhibition, they were much too erratic to determine whether the slope and intercept increased equally as is demanded by the theory. Another experiment was made in which special effort was taken to insure more uniform results but with little success. The data in Table I show that the velocity of the reaction is independent of the concentration of substrate within the range of variation employed—0.15 to 0.8 atm. When the reciprocals of the k values were plotted against the proper $1/pN_2$ values, the fit of the points to a straight line was very poor so that estimates of both the slope and intercept were subject to rather large error. The intercepts increased significantly as required by non-competitive inhibition, but the errors in the slopes precluded any definite decision with respect to these constants. None differed significantly from zero, but neither did they differ significantly from other possible theoretical values.

The plant experiments were then abandoned in favor of studies on the free-living nitrogen-fixer *Azotobacter vinelandii.* Not only could much greater accuracy be obtained in the micro-respiration experiments made with this organism, but because of the time required the numerous trials which we shall see are necessary for adequate test of the kinetic equations could be completed in a reasonable period.

TABLE I

Non-Competitive Nature of Inhibition of N_2 Fixation by CO (Red Clover Experiments)

CO atm.	k values ($\times 10^3$) at different pN_2: 0.8 atm.	0.4 atm.	0.2 atm.	0.15 atm.	Intercept*	Slope*
0.0000	5.66	6.65	5.36	5.43	17.91	+0.037
	5.37	5.62	5.00	6.16	±1.00	±0.231
	5.90	4.51	6.06	5.26		
	5.72	5.51				
Relative k	*100*	*100*	*100*	*100*		
0.0001	4.04	4.48	4.22	5.07	22.84	−0.261
	4.89	4.83	4.94	4.07	±1.46	±0.345
	3.99	4.79	5.37			
Relative k	*75*	*84*	*88*	*81*		
0.0002	4.15	2.17	2.82	2.98	32.88	+0.414
	2.54	2.79	3.85	2.43	±4.20	±0.954
	3.79	2.66	2.73	3.16		
Relative k	*62*	*46*	*57*	*51*		

* In tables I, IV, and V, the value given for a slope (or intercept) is the "best" estimate ± its standard deviation calculated by the usual methods of statistics. For details of such calculations see Eisenhart and Wilson (10).

Azotobacter Experiments

Initially it was planned to vary the pN_2 from 0.1 to 0.8 atm. The upper limit chosen is the pN_2 of air,[3] and since the rate of fixation is

[3] Actually 0.78 atm., but for convenience we shall write 0.8 atm. in this paper. In the calculations, however, the true value was used.

TABLE II

Type of CO Inhibition of Nitrogen Fixation by Azotobacter

Expt. No.	pN_2 *atm.*	CO *atm.*	k values × 10 *Actual*	*Relative*	k/k_i
1	0.8	0.000	3.31	100	1.000
		0.002	3.07	93	1.080
			2.83	85	1.170
		0.004	2.61	79	1.268
			2.26	68	1.466
	0.1	0.000	2.53	100	1.000
			2.65		
		0.002	2.40	93	1.079
			2.42	93	1.070
		0.004	1.51	58	1.718
			1.60		
2	0.8	0.000	3.32	100	1.000
		0.002	3.00	90	1.105
			3.22	97	1.103
		0.004	2.43	76	1.365
	0.1	0.000	2.52	100	1.000
			2.53		
		0.002	2.12	84	1.192
			2.00	79	1.262
		0.004	1.53	61	1.655
3	0.8	0.000	3.62	100	1.000
		0.002	2.55	71	1.420
			2.75	76	1.315
		0.006	1.58	41	2.290
	0.1	0.000	3.20	100	1.000
			3.40		
		0.002	2.54	77	1.300
			2.66	81	1.240
		0.006	1.51	46	2.182
			1.30	39	2.538

90–95 per cent of the maximum at this concentration of substrate, further increase was hardly necessary. The lower limit was taken at 0.1 atm. to avoid errors arising from lack of exact control of the composition of the gas mixture. As will be discussed later, even minor errors in making up the gas mixture may be quite important when the pN_2 used is less than 0.1 atm. Results from three typical preliminary

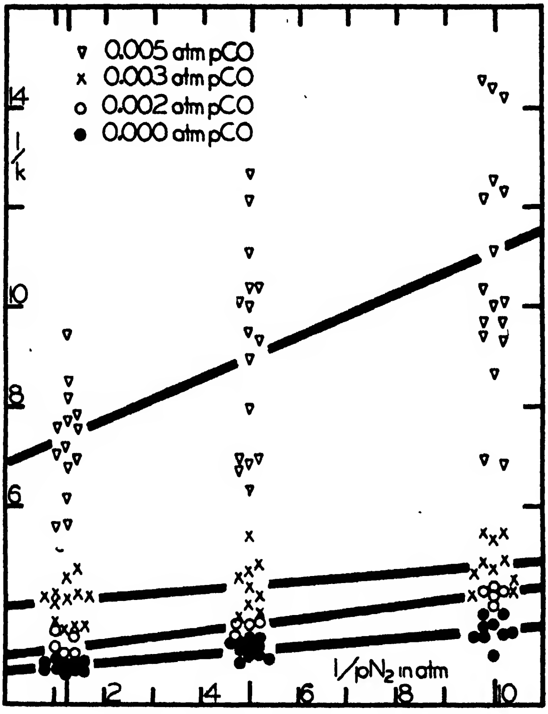

FIG. 1

Double Reciprocal Plot of Data from *Series I* of *Azotobacter* Experiments
Individual observations shown. pN_2 = 0.1–0.8 atm.

experiments are given in Table II. Apparently the percentage inhibition does not depend on the pN_2, but it is difficult to say just how close the agreement should be in order to claim complete independence.

Series I. On the basis of the results from the preliminary trials given in Table II, a large number of experiments were made in which the pCO was kept constant at various levels and the pN_2 varied. The

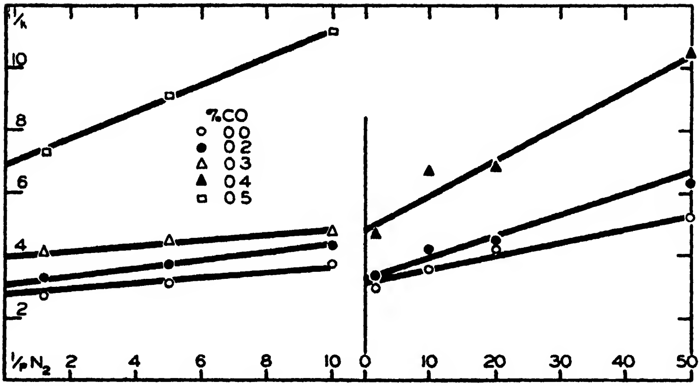

FIG. 2

Nature of the CO Inhibition of Nitrogen Fixation by *Azotobacter*

Double reciprocal plot according to Equation 12. In Figs. 2, 4, and 6, mean values only of replicate points are shown. Left: *Series I;* Right: *Series III.*

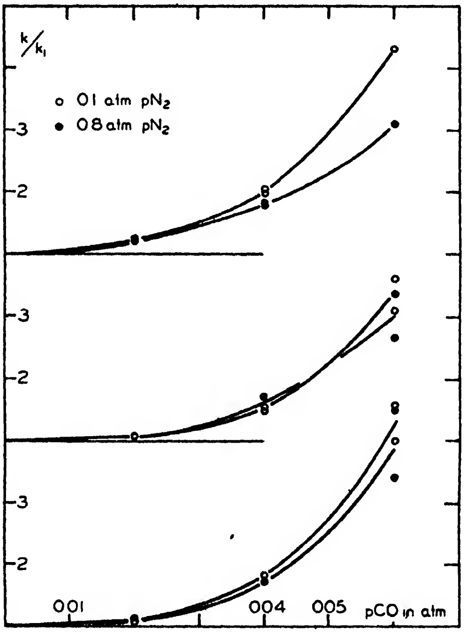

FIG. 3

Typical Results from Three Experiments in *Series II* in Which the Relative Rate of Nitrogen Fixation by *Azotobacter* is Plotted Against Concentration of Inhibitor

Individual values shown. Note that effect is independent of substrate concentration.

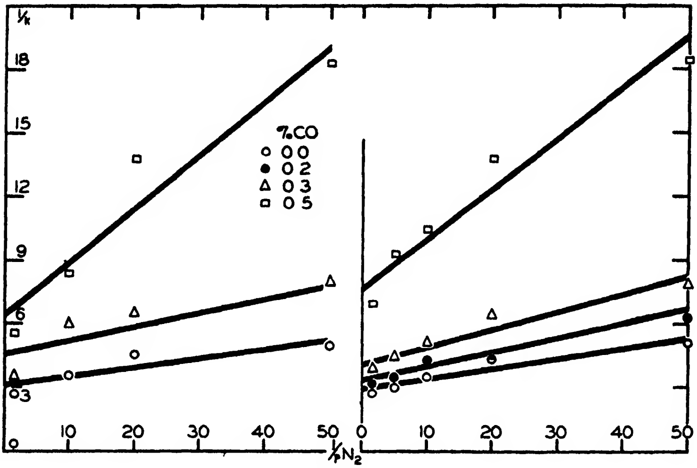

FIG. 4

Further Tests on Nature of CO Inhibition of Nitrogen Fixation
Left: *Series IV;* Right: Combined data from Series *I*, *III*, and *IV*.

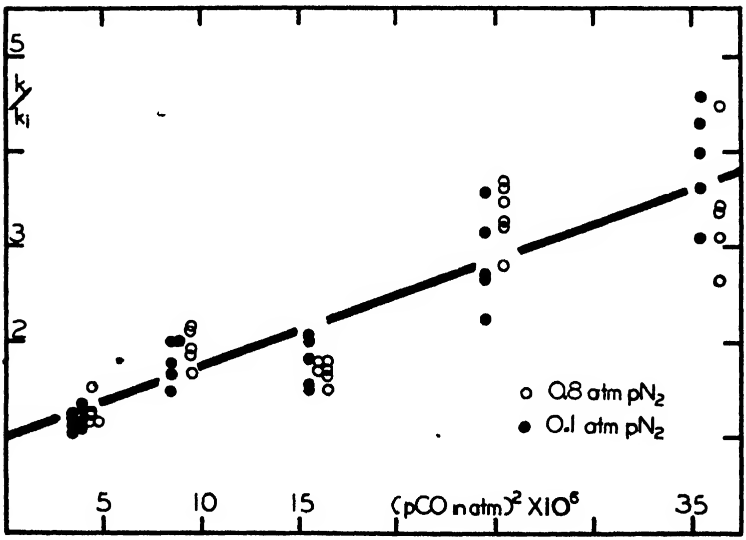

FIG. 5

Results from *Series II* Plotted According to Equation 13′ with $r = 2$
Individual values given. As in Fig. 3 the lines formed are independent of substrate concentration.

slopes and intercepts were then calculated for the lines obtained when $1/k$ was plotted against $1/pN_2$. The results are shown in Figs. 1 and 2.

Series II. This was similar to *Series I* except that the pN_2 was kept constant at 0.8 and 0.1 atm. and the pCO varied. The data were then plotted according to Equations 7 and 13 (Fig. 3).

Series III. Consideration of the data from *Series I* and *II* led to the conclusion that the range of concentration of substrate would have to be extended for final decision as to type of inhibition. In the remaining experiments the pN_2 was varied from 0.02 to 0.8 atm. Special precautions were taken to control the composition of the gas mixtures. These were prepared in 10 liter serum bottles which were evacuated until the desired pN_2 remained, then the pO_2 made to 0.2 atm. Helium was supplied to atmospheric pressure; the nitrogen in the helium was considered in preparing the mixtures. For the lowest pN_2 used, 0.02 atm., 0.8 atm. of tank helium was added to 0.2 atm. O_2. Analyses by the isotopic dilution method indicated that the helium contained 2.5% N_2, a value in excellent agreement with that previously found (11). The respirometer vessels were evacuated to about 4 cm. Hg residual pressure, then the required gas mixture added; this was repeated three times. The gas mixture was transferred from its container to the vessel by a Urey pump to avoid use of water as a displacing fluid.

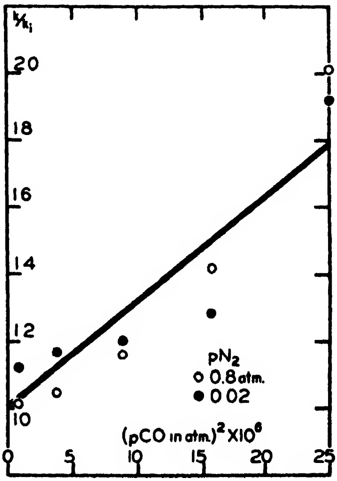

FIG. 6

Effect of Substrate Concentration (pN_2) on CO Inhibition of Nitrogen Fixation by *Azotobacter*

Mean values of *Series V* plotted according to Equation 13′ with $r = 2$.

Series IV. This differed from *Series III* only in the levels of CO employed. The data from *Series III* and *IV* are plotted in Figs. 2 and 4.

Series V. This was a repetition of *Series II* except that a pN_2 of 0.02 atm. was used instead of 0.1 atm. Also, seven to eight readings were taken instead of the usual five in order to increase the accuracy of the estimates of the relatively small k values observed when the pN_2 is so low. The data are given in Fig. 6 and Table VI.

Mathematical Treatment of Data

Statistical Tests

Before discussing and interpreting the data, certain observations concerned with their accuracy and limitations may be useful for their evaluation. The velocity of the reaction was measured by the specific rate constant of nitrogen fixation, k. This constant is determined by plotting the log O_2 consumed per hour against time and estimating the slope of the "best" straight line by the usual statistical method (10). The slope times 2.303 equals k. Not only does this provide an unbiased estimate of k but also an estimate of its error (see Table III). From several years of experience in this laboratory it has been determined that under normal conditions the error of a k value of 0.30–

TABLE III

Summary of Statistical Tests

pN_2 *atm.*	pCO *atm.*	Number of tests (n)	Mean $\bar{k}$ value	Error $\bar{s}_k$*	$\bar{s}_k/\bar{k}$ *per cent*	Relative k value† *per cent*	k/k_i†
0.8	0.000	15	0.352	0.031	8.8	100	1.000
	0.002	12	0.298	0.022	7.4	85	1.180
	0.003	15	0.229	0.029	12.7	65	1.536
	0.005	19	0.130	0.033	25.4	37	2.708
0.2	0.000	9	0.322	0.023	7.1	100	1.000
	0.002	5	0.287	0.017	6.0	89	1.122
	0.003	9	0.231	0.027	11.7	72	1.394
	0.005	16	0.115	0.033	28.7	36	2.800
0.1	0.000	15	0.290	0.020	6.9	100	1.000
	0.002	14	0.245	0.029	11.8	84	1.183
	0.003	16	0.199	0.030	15.1	69	1.458
	0.005	21	0.094	0.035	37.2	32	3.085

* If the estimated standard deviation of the slope (b) of a given line is s_b, then $s_k = 2.303\ s_b$ since $k = 2.303\ b$. The s_k values of the lines corresponding to a given treatment, *e.g.*, a pN_2 of 0.8 and a pCO of 0.002 atm. were shown to be homogeneous (10), hence they could be combined and an average estimate adopted. These average estimates are given in Column 5 (denoted as average by bar over $\bar{s}_k$). Note that these average values must be divided by $\sqrt{n}$ (Col. 3) to obtain the standard deviation of the mean k value. Details of the necessary calculations are given in Reference 10.

† Average of n values.

0.40 based on 5 readings of *Azotobacter* growing in air ranges from 0.020 to 0.030, or roughly about 7%. This error, which measures the closeness of fit of the observed points to the best straight line that can be drawn through them, is relatively small. With such an error, the fit of the points is sufficiently close that deviations appear to be very slight, and the casual observer might be inclined to assume that the k value is known with a very high degree of accuracy. Whether this is true will depend on how important an error of this magnitude becomes for the purpose at hand.

Statistical analyses of a large number of trials summarized in Table III demonstrated that the error was about that usually obtained, but that the absolute value of the error did not decrease with a fall in the value of k. When the pCO approached that necessary for complete inhibition, the error actually increased since oxygen consumption by the organism under these conditions is small and somewhat erratic. Consequently, at a given pN_2, the k values from different trials will cluster together if the values are high (low pCO), but the scatter will be much more pronounced when low k values are obtained with a higher pCO. Likewise, decreasing the pN_2 will result in a wider range of k values.

When the reciprocals of these k values are taken, the scatter is even more outstanding. For example, if an average error of 0.020 is obtained in a series of trials, this means that in air (pN_2, 0.8 atm.; pCO, 0.0 atm.) the observed k values might range from 0.26 to 0.34. At the same pN_2 and a pCO of 0.005 atm. k values from 0.06 to 0.14 might be observed. In either case the range is 0.08. If, however, reciprocals of these are taken, the first values will fall between 2.94 and 3.84, a difference of 0.9, whereas the second set of values will be scattered from 7.1 to 16.7, a difference of 9.6. It is not surprising, then, that even though the inhibition is strictly according to one of the hypotheses, the fit of points of the "double reciprocal" plotting to the theoretical straight line may not appear to be too good especially at the high levels of inhibition.

Turning now to the variable which is considered to be known without error, it is seen that the result of reciprocal plotting may reveal in its true light what appears to be only a minor error in technic. For example, if the limit of control of the pN_2 is 0.01 atm. for partial pressures above 0.1 atm. and 0.005 atm. for those below, the value of $1/pN_2$ for an estimated 0.8 atm. might vary from 1.23 to 1.26, a negligible error. For a pN_2 of 0.02 atm., however, the actual value of $1/pN_2$

estimated to be 50, may be from 40 to 66.7. The same reasoning applies to the errors in the k/k_i type of plotting although the effect of errors in control of the variable in this case (pCO) is not so exaggerated since the reciprocal of (I) is not taken.

To minimize the effect of these errors we have employed two experimental devices: (a) in a given series of experiments the gas mixtures were prepared several times in order to "average out" errors in the control of the partial pressures of the critical gases; (b) all experiments were replicated several times to provide sufficient estimates of a given point that its *mean* might be a fairly stable value even though the scatter of the individual estimates was rather pronounced. In this manner not only was the nature of the inhibition established but also enough data were obtained to provide a satisfactory quantitative test of the implications of the kinetic equations. Agreement with these furnishes indirect, though by no means conclusive, support for the adequacy of the enzyme mechanism postulated.

The magnitude of the errors and the efficacy of the methods taken to control them are illustrated in Figs. 1 and 2. In the first figure the individual values of $1/k$ are shown, and it is evident that the range about the line increases as the pCO is raised. In Fig. 2 the mean values only of these individual estimates are plotted, and it is seen that they fit the line very well. Nevertheless, the scatter of the individual values must be considered, and as will be noted later, this is reflected in the errors attached to the estimates of the slope and intercept.

The Dissociation Constant

The chief criterion for testing whether the results are in quantitative agreement with the kinetic equations has been the constancy of K_{N_2}, measured by slope/intercept. The value of this criterion depends upon what shall be regarded *a priori* as a likely estimate. Using data from a variety of experiments we have previously suggested 0.02 ± 0.005 atm. (11). This estimate receives confirmation from the relevant data obtained in these experiments. Calculations based on the k values obtained in the absence of CO (see Formula 4, Ref. 11), gave the following results: If the mean of 26 values obtained with a pN_2 of 0.8 atm. in *Series I* and *II* (0.346 ± 0.0055) is compared with that of 12 values observed with a pN_2 of 0.21 atm. (0.322 ± 0.0065), a K_{N_2} value of 0.022 ± 0.0084 is indicated. If the 0.8 atm. values are compared with the 18 contained at a pN_2 of 0.10 atm. (0.291 ± 0.0053),

the dissociation constant is calculated to be 0.0274 ± 0.0039 atm. In the 0.02–0.8 atm. series 18 different estimates from 9 separate experiments were available. These gave a value of 0.0215 ± 0.0007. It was concluded that variations of the constant between 0.015 and 0.025 are without significance and that variations between 0.010 and 0.030 are only suggestive.

Discussion

Over 40 experiments have been completed in which the pN_2 and the pCO have been varied and the results subjected to the necessary mathematical analysis. The relevant data are shown in Figs. 1–6, and the statistical constants summarized in Tables IV–VI. These data can be most readily interpreted by considering them according to the method of plotting.

TABLE IV

Statistical Constants for Data Given in Figures

Series	Range of pN_2 *atm.*	pCO *atm.*	n	Slope	Intercept	K_{N_2}
I	0.1 –0.8	0.000	29	0.085 ± 0.013	2.74 ± 0.082	0.031
		0.002	15	0.126 ± 0.013	3.01 ± 0.088	0.042
		0.003	32	0.074 ± 0.022	3.97 ± 0.146	0.019
		0.005	48	0.429 ± 0.083	6.83 ± 0.567	0.063
III	0.02–0.8	0.000	44	0.046 ± 0.005	3.03 ± 0.148	0.015
		0.002	18	0.069 ± 0.011	3.19 ± 0.335	0.022
		0.004	21	0.117 ± 0.014	4.68 ± 0.406	0.026
IV	0.02–0.8	0.000	17	0.041 ± 0.010	3.12 ± 0.299	0.013
		0.003	15	0.072 ± 0.025	4.55 ± 0.568	0.016
		0.005	14	0.255 ± 0.088	6.27 ± 2.635	0.041
I, III, IV	0.02–0.8	0.000	90	0.047 ± 0.004	2.95 ± 0.085	0.016
		0.002	33	0.069 ± 0.007	3.26 ± 0.162	0.021
		0.003	47	0.083 ± 0.011	4.03 ± 0.208	0.021
		0.005	62	0.237 ± 0.038	7.64 ± 0.564	0.031

$1/k$ vs. $1/pN_2$. The data from *Series I* (Figs. 1, 2; Table IV) point to definitely non-competitive inhibition with possibly a suggestion of competitive inhibition. The intercepts increase consistently with rise in pCO with a corresponding increase in the slope except in the set in which the pCO equals 0.003 atm. Even in this case, if the errors in

the constants are considered, the failure of the slope to increase according to the theory may be apparent rather than real. This is borne out by the calculated values for the K_{N_2}. That found in the absence of CO is somewhat, but not unduly, higher than usual (11). About the only conclusion is that the observed dissociation constants for the 0.000, 0.002, and 0.003 atm. sets do not differ significantly. The value obtained in the 0.005 atm. set is undoubtedly high, but it must be remembered that this is the one with least precision so that taken by itself this apparent evidence for competition is not too convincing.

In *Series III* and *IV* (Figs. 2, 4; Table IV) the range of substrate concentration, and therefore of k values, was considerably enlarged so that these data may be regarded as perhaps of greater reliability than those of the first series. Both slope and intercept increased with concentration of inhibitor in agreement with the theory of non-competitive inhibition. The slope, however, increased somewhat more than did the intercept as is evidenced by the small but consistent rise in the K_{N_2}. That the same small but definite rise is observed when the data from all experiments are combined is even more impressive. Because of the increased number of points available in the combined data, both intercept and slope have greater precision. Hence, the small increase in K_{N_2} with the pCO might be important—reflecting the occurrence of some, though not pronounced, competitive inhibition.

k/k_i vs. pCO. - When, in *Series II* and *V*, k/k_i was plotted against the pCO, the resulting lines were concave upward curves (Fig. 3). The usual interpretation of such a result is that the inhibitor enters Equation 13 as a power greater than one. In derivation of the equations it was assumed that one molecule of inhibitor combines with one molecule of enzyme (prosthetic group) since this assumption possesses a certain logical basis. It is recognized, however, that the assumption may not always be true. If r molecules of inhibitor combines with one of enzyme, Equation 13 becomes:

$$\frac{v}{v_i} = 1 + \frac{(I)^r}{K_i} \tag{13'}$$

from which

$$\log (v/v_i - 1) = \log 1/K_i + r \log (I). \tag{18}$$

The value of r is readily determined by plotting $\log (v/v_i - 1)$ against $\log (I)$ and estimating the slope of the resulting line (12). Calculation

of r according to equation 18 gave the results in Table V which indicate a value of 2.

As illustrated in Figs. 5 and 6 when the values from *Series II* and *V* are plotted against $(p\text{CO})^2$, the points fall along a line with unit intercept although the fit is not particularly good in Fig. 6. The data in Fig. 5 (as well as in Fig. 3) are unequivocally in favor of the view that the inhibition is non-competitive, *i.e.*, the slope for the line corresponding to a pN_2 of 0.1 atm. does not differ significantly from that of the 0.8 atm.

TABLE V

Estimation of r and $1/K_{CO}$ in Equation 18

Type of Experiment	No. of Experiments	Slope (r)	Intercept $\log 1/K_{CO}$	$1/K_{CO}$ *atm* $^{-2}$	Reference
Clover plants	9	2.11 ± 0.20	7.5370 ± 0.725	34.4×10^6	(1)
Azotobacter macro	9	2.19 ± 0.27	4.5476 ± 0.434	3.5×10^4	(2)
Azotobacter micro	17	2.19 ± 0.17	5.3651 ± 0.482	23.2×10^4	Series II
Azotobacter micro	15	1.90 ± 0.15	3.6563 ± 0.601	0.5×10^4	Series V

TABLE VI

Effect of pN_2 on Value of k/k_i (Series V)

$p\text{CO} \times 10^3$ *atm.*	$pN_2 = 0.8$ atm. n	$pN_2 = 0.8$ atm. k/k_i	$pN_2 = 0.02$ atm. n	$pN_2 = 0.02$ atm. k/k_i
1	5	1.012 ± 0.019	6	1.120 ± 0.036
2	7	1.048 ± 0.024	11	1.170 ± 0.027
3	8	1.175 ± 0.046	12	1.200 ± 0.029
4	8	1.420 ± 0.077	11	1.280 ± 0.030
5	6	2.015 ± 0.290	9	1.920 ± 0.240

line. One line was therefore fitted to all points independent of the pN_2, and a slope of 7.43×10^4 calculated.

The data from *Series V*, which are shown in Fig. 6, point once again to the possible existence of a small but definite competitive inhibition in addition to the predominant non-competitive. Low concentrations of CO inhibit more effectively at a pN_2 of 0.02 atm. than at 0.8 atm. as is shown by the statistical analysis in Table VI. At a pCO of 0.003 atm. or more, the values of k/k_i are identical within the rather large experimental error. Likewise, when the data are considered as a whole by determination of the line, strictly non-competitive inhibition is indicated with no admixture of the competitive type. The slope of the

0.02 atm. line in Fig. 6 was the same within experimental error as that for the 0.8 atm. line. Their common slope was determined as 3.14×10^4.

An alternative explanation of the curves shown in Fig. 3 is afforded by Winzler's recent analysis (6) of the action of specific inhibitors on the respiration of yeast cells. In a significant paper for this field he points out that although v in Equation 13 is usually measured directly by determining the velocity in the absence of inhibitor, this procedure may not always be correct. If the normal rate-limiting reaction in the chain which constitutes the process being measured is not the one affected by the presence of inhibitor, the observed maximum rate of reaction (when $(I) = 0$) is not identical with the theoretical maximum. The latter is estimated by the true intercept of the line formed when v/v_i is plotted against (I), *i.e.*, the intercept is not 1.00 as it is drawn in Fig. 3. When the best line was determined for the data of *Series II* plotted in this manner, the fit of the points was very poor. The value for the intercept, 0.025 ± 0.175, is obviously too inaccurate to draw any conclusions about the rate-limiting reaction other than that it is apparently not the one inhibited. The data from *Series V* gave the more reasonable and accurate value of 0.72 ± 0.083. In either case, whether k/k_i is plotted against $p\text{CO}$ or $(p\text{CO})^2$, the lines corresponding to different values of the pN_2 are the same within experimental error.

Consideration of all the data leads to the conclusion that carbon monoxide inhibition of biological nitrogen fixation is primarily non-competitive. There is some evidence, however, that the predominantly non-competitive inhibition contains elements of competitive, *e.g.*, the affinity of CO for E is greater than for ES, or the dissociation constant is increased because of the presence of CO on the enzyme (see Winzler, 6, for a similar observation concerned with the action of cyanide on Atmungsferment).

Turning to the other possibilities, it is evident that there is no reason to suspect Type IV ("uncompetitive") since the slope increases with rise in $p\text{CO}$ in practically all sets. Likewise, little evidence for the existence of Type V ("quadratic") was obtained. This was somewhat surprising since nitrogen fixation is certainly not a simple process, and *a priori* one might suspect a mechanism in which the inhibitor affects a reaction somewhat removed from the primary one involved in the first step of fixation. In spite of its logical attractiveness, however, no support for this conclusion was uncovered. Although the slopes of the lines

in the 0.1 to 0.8 atm. series are small and might be reasonably regarded as "practically" zero, it is seen that if sufficient determinations are made, definite positive values are obtained consistent with independent estimates of the dissociation constant, K_{N_2}. Moreover, when the data from this series are plotted with those from the others of more extended pN_2 range, there is no sign of a discontinuous break in the curve which would suggest a change of slope from nearly zero to that of the line in the absence of CO. Instead, slopes uniformily greater throughout the entire pN_2 range than that of the 0.000 atm. line are obtained, and these increase with the pCO (Fig. 4).

Dissociation Constant of CO-Enzyme Complex. Equation 13′ states that the slope of the line is $1/K_{CO}$. Fig. 5 yielded a value of 13.4×10^{-6} for K_{CO}, Fig. 6, one of 31.8×10^{-6}. The former is in better agreement with our previous studies (2, 3) as it indicates that the enzyme is half-saturated with CO when the partial pressure of this gas is somewhat less than 0.004 atm. The line obtained when the k/k_i values of *Series V* were plotted against the pCO rather than the square of this concentration had a slope of 203 and an intercept of 0.72. These values correspond to a K_{CO} of 3.5×10^{-3} atm. (enzyme half-saturated at 0.0035 atm.). Another estimate of K_{CO} is based on Equation 18 since the intercept of the line in this equation is log $1/K_{CO}$. The values obtained, however, are rather inaccurate because of the relatively large error in the intercept (Table V). They may be regarded only as estimates indicating the order of magnitude and serve as a check of the more reliable values obtained by the other methods.

Because of the agreement among these estimates independent of whether the relative velocity constant is plotted against pCO or $(p\text{CO})^2$ one might be tempted to overestimate their significance. It is not at all certain that they actually are reliable estimates of a true equilibrium constant. Johnson, Eyring, and Williams (12) have shown, for example, that the intercept in Equation 18 represents a simple equilibrium constant only if the inhibitor combines indiscriminately with native and denatured forms of the enzyme. To decide this, extensive observations on how changes in temperature, or pressure, affect the activity of the enzyme are necessary.

Summary

Methods for determining the type of inhibition observed in an enzyme reaction are discussed, and a systematic procedure based on the graphical

methods of Lineweaver and Burk is outlined. Supplementing the graphical analysis, the advantages of statistical treatment of the data for final interpretation are noted.

The usefulness of the described procedure is illustrated by a detailed analysis of the type of CO inhibition in biological nitrogen fixation. Most of the data for this treatment were obtained with *Azotobacter vinelandii* in micro-respirometer experiments.

It is concluded that the inhibition is primarily non-competitive. There is also some suggestion of an accompanying competitive inhibition (*e.g.*, greater affinity of CO for nitrogenase than for the nitrogenase-N_2 complex). If the competitive type actually is present, it is quantitatively much less important than the predominating non-competitive type.

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The Biochemistry of Vibrio cholerae

I. Growth Methods

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Introduction

For studies on the chemical activities and immunogenic structure of *Vibrio cholerae* it is desirable to use a simple medium which will permit massive growth and at the same time will be readily eliminated after growth had taken place, leaving behind the bacteria and their soluble products of high molecular weight.

Hirsch (1) has described a medium consisting of salts and sodium asparaginate which sufficed for studies upon the metabolism of the vibrios and upon their growth products, but in our hands it failed to provide the massive growth which was one of the goals of our study. Linton, Shrivastava, and Seal (2) made use of eight media containing either peptone or mutton infusion with various amounts of inorganic salts and glucose. They concluded that the medium itself was a factor of outstanding importance in the production of polysaccharide. In general the polysaccharide was more abundant in the media to which no glucose had been added.

The use of peptone-glucose media introduces large amounts of foreign substance which might have an effect both in vaccination and in work on the chemical isolation of antigenic fractions. For this reason a number of changes and improvements have been made in the culture medium.

Development of a Suitable Medium

Originally the vibrios were grown in 2% peptone medium, adjusted to pH 8.0 and incubated for 72 hours. The peptone solution was obtained by dialyzing 1 part of 20% bacto-peptone solution (Difco) in Visking tubing against 9 parts of distilled water in the cold for 3 to 5 days, and using the dialyzate as the medium. It was hoped that after growth was completed, the medium itself could be com-

pletely dialyzed away. The color of the peptone, however, could not be removed by dialysis, and the growth was poor. Attempts were then made to improve the growth by adding glucose to the peptone medium with periodic adjustment of the pH by sodium bicarbonate. While this change led to a somewhat increased growth, no real improvement resulted, since the bulk of the medium was greatly increased by the large volume of bicarbonate solution.

Since the presence of 2% peptone seemed to make the isolation of the products of growth more difficult, it was decided to turn to a medium made up largely of inorganic salts with a minimum of nitrogenous matter:

$(NH_4)_2SO_4$	5.0 g.
K_2HPO_4	0.75 g.
$MgSO_4$	0.10 g.
Peptone	2.0 g.
Glucose	1.0 g.
Dist. water	to make 1 liter.

The first two salts are completely dissolved in about three-quarters of the liquid and the $MgSO_4$ in the remainder.

In cultures made in this medium, the growth was heavy, and all the sugar had disappeared within 48 hrs., the pH dropping from 8.3 to 5.35 during the same period. It appeared to make no difference in the final crop and in the final pH whether the glucose was added during growth or at the beginning; nor did it make any difference whether a 6, 18, or 24 hrs. culture was used for inoculation.

Large Volumes of Culture. As a final step, cultures were made in 12 liters of the salt medium with equivalent amounts of dilute peptone and sugar. The medium was tinted with *m*-cresol purple and sterilized for 1 hour at 20 lbs. pressure in a 22 liter round-bottom Pyrex flask. 24 g. of sterile glucose in a liter of water, 250 ml. of a vigorous 18 hour culture of the vibrio, and sufficient 25% NaOH to turn the indicator purple were added. Growth was generally slow, requiring 3 days for even a moderate production of vibrios, but occasionally a strain would grow rapidly during the first 24 hrs., turning the indicator yellow. When this occurred growth ceased and could not be revived by the addition of more alkali or nutrients or even of an actively growing fresh culture. The growth under these conditions, however, was as good as in 2% peptone water.

Casein Digest. A final change was introduced into the preparation of the medium by replacing peptone entirely by casein digest, prepared as follows: 100 g. of commercial casein were suspended in 1 l. of water and the pH adjusted to 8.0 to 8.5 with solid Na_2CO_3. One-half gram of trypsin (Difco) suspended in 10 ml. of water was added and the mixture kept at 37°C. for about 48 hours. The flask was shaken and the pH adjusted from time to time. After digestion, the pH was brought to 6.5 to 7.0 and the undissolved portion filtered off. The filtrate was stored over chloroform in the refrigerator.

To each liter of the salt solution 15 ml. of trypsin casein digest solution (C–D), having in the various batches between 9 and 12 mg. of nitrogen per ml., were added in place of the peptone. The planting culture of 80 ml. of the salt solution had an equivalent amount of the C–D medium added to it as well. In this way the highly colored constituents of peptone were avoided and a practically colorless

medium was made available. Doubling the amount of C–D had no beneficial effect upon the amount of growth.

Aeration. The use of such large amounts of medium involved a low surface-depth ratio, with the probability of reduced access of the organisms to air. It was therefore decided to stir the cultures by means of a current of gas.

Carbon dioxide was first bubbled through the medium for 10 to 12 minutes in each experiment, followed in the earlier experiments by nitrogen and in the later ones by air. Air was drawn from the compressed air supply of the laboratory, while the other gases were commercial products. The rate of flow was about 30 l. per hour.

Originally bubbling was achieved through a pointed glass tube reaching to the bottom of the flask. A 200 × 15 mm. $CaCl_2$ tube stuffed with cotton acted as an efficient air filter, since we never experienced contamination from this source. Later the bubbling tube was modified. The constricted opening was changed to a flanged end over which was tied a fairly large cloth bag, giving a mass of very fine bubbles rather than a single column of larger ones. Better growth was now obtained, the highest recorded for the 13 l. lots being 1750 p.p.m., while in the liter quantities the equivalent of 2000 p.p.m. was regularly reached.

Final Technique

Electrometric pH determinations showed that the salt medium had a pH of 7.4 before autoclaving, and of 6.8 after autoclaving. By experiment it was found that the addition of 3.3 ml. of 25% NaOH per liter brought the pH to 8.8, which appeared a suitable point to begin growth, and the addition of this amount of alkali was continued routinely.

Planting was done as follows: 30 g. sterile glucose and 43 ml. of 25% NaOH were added to 12 l. of the sterile salt medium in a 22 l. round-bottom Pyrex flask. After a thorough shaking to distribute the alkali, one liter of an 18 hour casein digest culture of the vibrio strain was likewise poured in and thoroughly shaken. The flask was then transferred to the incubator and CO_2 was bubbled through for 10 minutes followed by air or N_2. Certain of the results where air was used are given in the following table.

As the table shows, the final pH varied between 5.5 and 6.5, and there did not appear to be any correlation between this finding and the amount of growth. In fact, none of the factors could be correlated with the amount of final growth attained. When as in the case of strain 20, the culture was repeated a number of times, growth was variable and so was the terminal pH, indicating that some other factor or factors than strain differences were responsible for these variations. Consistent results followed the use of 30 g. of glucose (0.25%) although here again there was a variation in time, since one strain might take a longer period

than another to use this amount of sugar. The addition of 40 or 45 g. of glucose did not result in improved growth, and unless the growth period was extended beyond 24 hours some sugar remained to complicate the ensuing procedures. Cultures in the 22 l. flasks in the absence of aeration showed about 250 p.p.m. of growth. Accordingly, even the

TABLE I

The Effect of Aeration Upon Growth of Various Vibrio Strains in a Liquid Medium

Vibrio strain No.	Final pH	Glucose amount *g.*	Benedict's test at finish	Growth period *hrs.*	Growth* *in p.p.m.*
Control 1	5.5	30	4+	24	<250**
Control 2	6.0	30	3+	24	<250**
17	6.5	30	neg.	24	1000
25	5.5	30	neg.	24	500
13	5.5	45	sl. pos.	24	1000
27	5.5	45	neg.	48	1500
28	5.5	45	sl. pos.	30	1250
29	5.5	40	v. sl. pos.	30	500
30	6.5	30	neg.	24	1000
26	6.5	30	neg.	90	750
31	6.5	30	neg.	72	500
21	—	30	neg.	30	500
20-2	5.5	30	neg.	24	750
20-5	6.5	30	neg.	24	1000
20-6	5.5	30	neg.	31	1000
20-8	6.0	30	neg.	24	1250
20-9	5.5	30	neg.	24	1000
20-10	6.5	30	neg.	24	1250
20-11	6.0	30	neg.	24	750
20-12	6.5	30	neg.	24	1250
20-13	6.0	30	neg.	24	1000

* For the preparation of the silica standard used in this work, see American Public Health Association, Standard Methods—3rd Ed. Boston (1917).

** Growth unchanged at 72 hours.

worst growth with aeration was twice as good as when no air was passed in.

Further Investigations

Ammonium Sulfate. While the growth method which we have outlined was being carried out routinely for the production of vaccines and polysaccharides, further experiments were under way to see if improve-

ments could be made. The data from these experiments, all of which were carried out with aeration in 1 l. amounts of medium in three-liter flasks, have been collected in Tables II A and II B. The typical vibrio strain No. 35 was used throughout and planting was done with 18 hour cultures in 80 ml. of the C–D salt medium. These flasks were first used in a study of the effect of varying the amount of ammonium sulfate upon the growth and the pH.

TABLE II A

The Effect of Varying Amounts of Ammonium Sulfate Upon Growth of Vibrio 35

		pH readings				
Flask	$(NH_4)_2SO_4$ *mg.*	Before planting	After add'n of NaOH and glucose	After planting	After CO_2 2 min.	After incubation
34	0	8.2	11.7	11.5	10.85	24 hrs.—9.2 48 hrs.—8.1
26	5	8.1	11.5	11.2	—	7.3
30	5	8.1	11.65	11.5	10.4	6.9
27	50	8.6	11.6	11.1	—	8.3
31	50	7.9	11.5	11.4	10.05	6.9
28	500	8.2	11.4	10.9	—	24 hrs.—9.6 48 hrs.—8.6
32	500	7.0	11.4	11.1	9.6	24 hrs.—7.1 48 hrs.—8.5
29	2500	7.6	9.2	9.1	—	6.8
33	2500	7.3	9.2	9.15	8.8	7.5
35	5000	6.8	8.8	8.7	8.5	7.0
36	5000	6.85	8.8	8.7	8.4	24 hrs.—5.5 48 hrs.—5.1

Where the test for sugar was positive after 24 hrs., the incubation was continued for an equal period. When no time is stated, 24 hrs. is meant.

Tables IIA and B bring out the following points. In the first place, ammonium sulfate is not an essential part of the medium since growth will occur without it, as shown in flask 34 and again below in flasks 37–39 (Tables III A and B). The function of this salt is to act as a buffer. The addition of the planting culture has a small but constant acidifying effect upon the medium. The result of adding CO_2 to the flask is to lower the pH to a point where growth becomes possible when ammonium sulfate is absent or present in only small concentration. The CO_2

added to the medium containing the larger amounts of ammonium sulfate causes relatively little change in the pH, compared with that brought about when a small amount or none of this salt is present.

TABLE II B

Final Measurements

Flask	Period *hrs.*	Qualitative Benedict's Test	pH	Growth *in p.p.m.*
34	48	Negative	8.1	2000
26	24	"	7.3	1750
30	24	"	6.9	2000
27	24	"	8.3	2500
31	24	"	6.9	2000
28	24	Positive	9.6	0
	48	Negative	8.6	2000
32	24	Positive	7.1	2000
	48	Negative	8.5	2500
29	24	Negative	6.8	1250
33	24	"	7.5	2500
35	24	"	7.0	2500
36	24	Positive	5.5	1760

TABLE III A

The Effect of Varying Amounts of CO_2 Upon Growth and pH in the Absence of Ammonium Sulfate

Flask No.	CO_2 *min.*	pH readings: Before planting	After add'n of NaOH	After planting	After CO_2	After incubation (*24 hrs*)
37	3	8.0	11.6	11.4	9.85	6.9
38	6	8.05	11.65	11.5	7.1	7.85
39	9	8.0	11.65	11.4	7.2	7.5

TABLE III B

Final Measurements

Flask No.	Qualitative Benedict's test	Growth *in p.p.m.*	pH
37	Negative	1750	6.9
38	"	1750	7.85
39	"	2000	7.5

When the pH at the beginning of the experiment is high, the fall during growth is greater than when the initial pH is lower. This, of course, is a further expression of the activity of the ammonium sulfate.

There does not appear to be much connection between the final pH and the amount of growth obtained.

Since flask 34 in the above series had shown growth without ammonium sulfate in the medium, three more flasks were set up also without this salt to study further the effect of CO_2 upon the pH. The results are given in Tables IIIA and B.

When growth commencing at high alkalinity went to completion, *i.e.* used up a maximum amount of glucose, the culture still did not become acid. It was interesting to note that in flasks 38 and 39 there was an actual rise in pH during the growth, so that the final reading was more alkaline than at the beginning, although all the sugar had been consumed. This finding has been made also in one of the 12 l. cultures where an initial pH of 6.6 was raised to 7.75 while 30 g. of sugar were used up during a period of 24 hours.

Ammonium sulfate was retained in the medium for its buffering action, thus permitting the addition of an optimum amount of NaOH in order to provide a further neutralizer for the acids formed by the vibrios.

The influence of ammonium sulfate upon changes in the pH of media undergoing bubbling with CO_2 both with and without C–D or peptone is indicated in Table IV.

The first flask (No. 45) contained only $MgSO_4$ and K_2HPO_4 and showed a wide swing in pH both with alkali and CO_2 addition. With peptone added (No. 50) the combination had a greater rise but a smaller subsequent fall. With C–D, the rise in pH units was 1.95 and the fall 1.4, in contrast to the peptone-containing flask, where these changes were 4.0 and 2.7 or about twice as great. Accordingly, C–D was a much better buffer than peptone, in the absence of ammonium sulfate.

The three other flasks were similarly treated, but in the presence of ammonium sulfate. Here the changes were much less, although the presence of either peptone or C–D gave a somewhat better buffering action than the salt alone.

The salts together with glucose will not support growth. The addition of a few ml. of either peptone or casein digest solution, however, brings about maximum growth. There was no significant difference found in the pH curves with peptone or C–D. The advantages of using the C–D medium accordingly appeared to lie in its lack of color and its diffusibility through Visking tubing.

Glucose. The problem of maximum growth raised the question whether larger amounts of growth could be obtained with more glucose in a medium of maximum alkalinity. Many experiments were carried

out by raising the pH to 10 and adding up to 1% of glucose to the whole salt medium. The results may be briefly summarized in the statements that at a pH of around 10, growth sometimes occurred and sometimes did not, depending presumably upon small variations in pH; that at more alkaline pH readings, growth was not found to occur; and finally that no matter how high the pH (up to 10) or how much glucose was present, growth was not any better than when the pH was about 8.8–9.0 and 0.25% glucose was added. It appeared therefore that an

TABLE IV

The Influence of Ammonium Sulfate and CO_2 on Changes in pH of Uninoculated Media

Flask	Medium*	Initial pH	pH after 3.3 ml. 25% NaOH	pH after CO_2 for 1 min.	3 min.	5 min.	7 min.	9 min.	10 min.
45	Salts; No Amm. Sulf., No C–D or Peptone	8.83	11.86	11.6	9.85	7.4	6.7	6.48	6.4
50	Salts; No Amm. Sulf.; Peptone	7.75	11.75	11.55	11.3	10.97	10.07	9.48	9.05
49	Salts; No Amm. Sulf.; C–D	8.05	10.00	9.8	9.55	9.3	9.15	8.75	8.6
47	Salts.; Amm. Sulf.; No C–D or Peptone	7.65	9.35	9.25	9.15	8.89	8.65	8.4	8.19
48	Salts; Amm. Sulf.; Peptone	7.51	9.31	9.25	9.19	9.0	8.86	8.75	8.7
46	Salts; Amm. Sulf. C–D	8.12	9.18	9.15	9.00	8.9	8.72	8.57	8.5

* K_2HPO_4, 0.75 g., and $MgSO_4$, 0.1 g. per liter; ammonium sulfate, 5.0 g. per liter; C–D, 15 ml. per liter; and peptone, 1.6 g. per liter. (1-liter medium in 3-liter round-bottom flasks).

increased yield of organisms was not to be reached by experiments in this direction, the maximum growth being equivalent to 2500 p.p.m. in the 1 l. amounts of medium and 1500 p.p.m. in the 13 l. amounts, with neither being regularly attained.

Summary

The development of a simple liquid medium for obtaining large amounts of cholera organisms has been described. Variations in

amounts of the different constituents, in the concentration of glucose and in the initial pH have been studied, together with the effect of aeration. The largest growth was obtained upon the simplest medium, which consisted only of inorganic salts, glucose and dilute tryptic digest of casein. When aeration was practiced in this medium, using devices to obtain masses of small bubbles, the maximum growth occurred within 24 hours. After removing the organisms, the liquid part of the culture was readily freed from the inorganic salts and casein digest, leaving the soluble bacterial products of high molecular weight in pure form.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Biochemical Research Foundation of the Franklin Institute.
Note by the Director: The consideration of this contract was one dollar.

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The Biochemistry of Vibrio cholerae

II. The Influence of Environmental Factors on Growth

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Introduction

The evolution of a culture method which permits the production of a dense population of *Vibrio cholerae* in a large volume of liquid medium has been described in the first paper of this series (1). The medium employed is sufficiently simple to permit its subsequent removal by dialysis and evaporation, leaving the vibrios and their growth products for use in preparing vaccines, antigenic fractions, or bacterial polysaccharide as desired. Details of method and reasons for the steps taken are presented here as a separate communication since they have some interest, independent of the development previously described, in revealing the behavior of the vibrio in response to its environment. The work reported in this paper, however, was done with the main object of developing the cultural technique and has not been carried to a greater length than will serve this purpose. Since the research was only intended to provide a structure of orientational hypotheses, less exact methods have been employed than may be necessary for positive proof for many of the conclusions. However, the hypotheses evolved have proved sufficiently correct to bring about notable improvements over previous cultural methods.

The studies reported here concern the response of the organisms to the presence of glucose, of acid, of ammonium sulfate, and of casein digest in the medium, and to certain gases as atmospheres for the culture. The latter were supplied by bubbling the gas through the culture and the effect of bubbling had to be considered both as a chemical and as a mechanical procedure.

Materials and Methods

The basic medium employed consisted of 0.5% ammonium sulfate, 0.075% dipotassium phosphate, and 0.01% magnesium sulfate in distilled water. To this solution was added 15 ml. per liter of a casein digest prepared by subjecting a 10% casein solution to tryptic digestion as described in the previous paper (1). This solution was sterilized by autoclaving and various concentrations of glucose, having been sterilized separately in concentrated solution, added as desired. Inoculation was made with cultures of a single representative strain of *Vibrio cholerae* grown in the same medium. This strain was chosen because of its particular interest as a satisfactory source of vaccine, as well as because it seemed to be among the more reliable of the strains available from the standpoint of growth. As a rule about 80 ml. of a 12 to 24 hrs. culture were used as an inoculum.

The cultures were usually made in a liter of medium contained in a three liter round-bottom flask. Just before inoculation the medium was brought to the desired alkalinity by the addition of 25% NaOH. For routine work, 3.3 ml. of this solution provided a satisfactory initial pH without further attempts at adjustment.

Variations in pH were followed by means of glass electrodes on samples removed aseptically at desired intervals. Sugar analyses were carried out on the same samples by the Folin-Wu colorimetric technique. An estimate of the growth was obtained by comparison with barium sulfate standards for turbidity. Later these were replaced with similar standards of sodium silicate (2). The composition of the gases used for bubbling was roughly regulated by simple flowmeters of the capillary type.

Problems Studied

The Influence of Glucose. Hirsch (3) has made a rather thorough study of the fate of glucose under specified conditions in cholera cultures. He stated that acid was formed in varying amounts according to whether the organism was growing aerobically or anaerobically. Under the conditions of our experiments it was quite obvious that acid was being formed. Reference to Fig. 1, representing the pH changes, the sugar content, and the turbidity in a typical culture, will confirm the close correlation between glucose metabolism and the pH, as well as the rate of growth of the culture. Both the pH and the glucose concentration start to decline as growth begins, fall off at a maximum rate during logarithmic growth, and reach a constant level at the end of active reproduction.

By varying the initial concentration of glucose it was possible to demonstrate that the higher the concentration of glucose, the more rapid and prolonged was the growth. The upper limit of this relationship appeared to be in the neighborhood of 0.3% glucose. This also repre-

sented the maximum amount of glucose that would be used by the organisms under the conditions of our experiments, even when an excess of glucose was available.

According to Hirsch's conclusions (4), under the conditions of our experiments we should expect the greater part of the glucose to be fer-

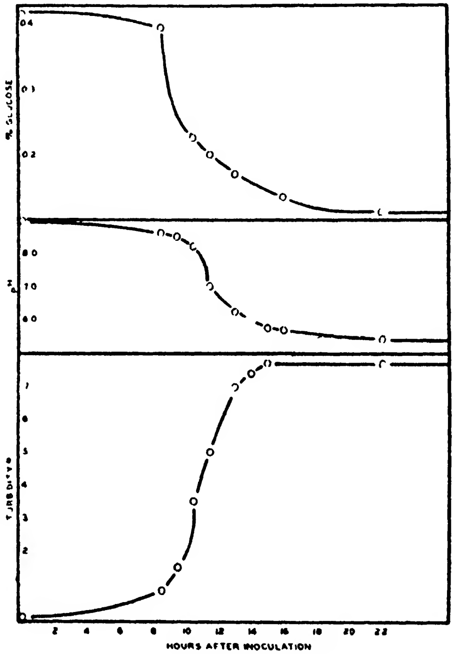

FIG. 1

The Relationship between the Rate of Glucose Utilization, Acidification and Growth during the Development of a Typical Culture in the Dialyzable Salts-Casein Digest Medium

* Turbidity is expressed as the number of the $BaSO_4$ standard which corresponds to the turbidity of the culture. The standards are prepared so that each represents approximately a tenfold increase.

mented with the formation of small amounts of ethyl alcohol and larger amounts of formic, acetic and lactic acid. The remainder of the glucose should be respired. The fate of the glucose was not thoroughly investigated as it did not appear to have any important significance for the main purpose of our work. Duclaux determinations, however, seemed

to indicate that the volatile acid present might be accounted for entirely as acetic acid. Lactic acid and alcohol have been detected.

It may be concluded that glucose serves as an important source of the energy needed for reproduction, and that it is utilized in a manner which results in the accumulation of acid in the medium. The organisms are capable of using about 3 grams of glucose per liter before growth is stopped.

The pH Changes During Growth. It has become customary to think of the pH optimum for the cholera organism as abnormally high. The unusual ability of the vibrio to survive exposure to rather strong alkali has been useful in isolating the organism from mixed cultures. Moreover, it is true that an initially high pH favors a maximum final crop of vibrios because of the increased amount of acid that may form before growth is inhibited.

The idea that alkalinity is desirable for the growth of *Vibrio cholerae* is acceptable, however, only if it is borne in mind that this condition is exceedingly transitory, and not representative of the conditions ideal for growth and reproduction. It may be seen from Fig. 1 that during the most rapid growth the pH was changing over the range from about 8.5 to 6. Within this range it cannot be said that alkalinity favored the development of the vibrio. Higher (and lower) values were associated with actually inferior growth rates.

In the experiment cited in Fig. 1 it appears that it might be equally true to state that slow growth was a corollary of high or low concentration of glucose. However, while it was true that with a low initial glucose concentration, growth was not as rapid, a medium containing originally a 1% glucose concentration gave rise to a growth and pH curve quite similar to that shown for the 0.4% concentration, and more glucose was left at the end than was originally present in the experiment represented Fig. 1.

These considerations would indicate that while a high initial pH may be optimal in the sense that it gives the best conditions for an extended period of growth the most desirable pH for rapid multiplication may lie in the region near neutrality. Experiments showed that a pH of 10 was injurious to the vibrio and usually prevented growth altogether. Invariably growth stopped when a pH of 5.5 was attained, whether at the end of a long vigorous growth starting at high pH or at the end of a shorter period when growth was initiated at a lower pH level.

Hirsch reported that the addition of calcium carbonate to his medium

resulted in a longer growth period. In the new medium, however, the addition of calcium carbonate seemed to have little effect on the culture. In the conditions imposed on the vibrio by Hirsch's experiments growth was much slower than in ours. Perhaps the calcium carbonate was not sufficiently active to stabilize the pH under the rapidly changing conditions in our cultures.

We were similarly unsuccessful in securing any greater crops by periodic neutralization of the medium with alkali during growth. It may very well be that the addition of base directly to the culture results in a local highly alkaline environment for many of the organisms in the culture before the medium as a whole has assimilated the added caustic, and that the injury sustained by these individuals more than counterbalances the benefit to the total population. Preliminary experiments on the effect of very short exposures to a high pH confirm the possibility of such injury.

If these explanations hold true it should follow that the buffer value of the medium would be all important in determining the final crop. Within certain limits this has proved to be the case. The buffering systems present in the original medium were found to be extremely important in producing satisfactory growth and will be discussed more thoroughly in a later section. The addition of fairly large amounts of phosphate buffer did not, however, give satisfactory results. In passing it may be well to state that a much more satisfactory means of stabilizing the pH has been found and is now under study. The results of this work will be published in a later paper in this series.

Ammonium Sulfate as a Buffer. Ammonium sulfate was originally incorporated in the medium as a possible source of nitrogen. Experiments in which the ammonium sulfate was omitted from the medium indeed supported the idea that the material was an essential nutrient. Subsequent study showed, however, that when ammonium sulfate was omitted, or when it was replaced by sodium sulfate, the initial pH of the medium was much too high to permit growth. When this condition was remedied by a corresponding decrease in the amount of sodium hydroxide, growth occurred but was unsatisfactory, due to an early attainment of the critical pH of 5.5.

In the previous paper (1) it was indicated that the presence of ammonium sulfate greatly increased the amount of CO_2 which the medium could neutralize. It will be seen from Fig. 2a that the medium at the time of inoculation did possess a buffering system operating largely

in the region of the initial pH near 9. The addition of sulfuric acid in small increments to a sample taken at the time of inoculation showed that the fall in pH was greatly restrained until acid equivalent to about 0.15 ml. of normal acid had been added. Similar titrations on samples taken at intervals during growth showed, as might be expected, that the buffering power had been depleted as though by the addition of acid, and that there were minor changes in the titration curve perhaps attributable to the formation of buffer from products of growth.

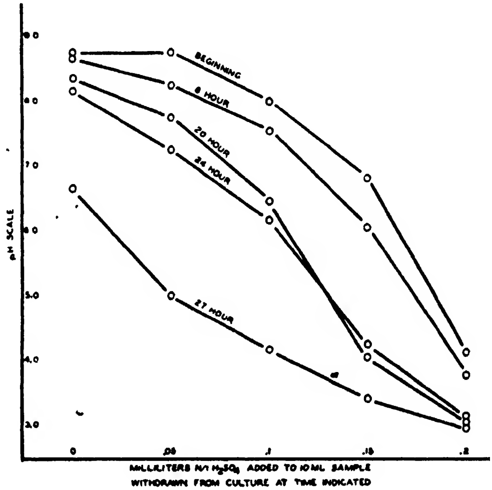

FIG. 2a

Changes in the Buffering Capacity of the Usual Medium during the Growth of *Vibrio cholerae*

The medium contains 5 g. of $(NH_4)_2SO_4$ per liter

The absence of ammonium sulfate resulted in a much more rapid depression in pH with the same additions of acid. In an experiment in which a minimum amount of $(NH_4)_2SO_4$ (5 mg./l.) was used in the medium, the curves obtained by similar titration were extremely steep and showed clearly the absence of buffering capacity (Fig. 2b).

Perhaps it should be mentioned that cultures differed greatly in the length of time which elapsed between inoculation and the initiation of rapid growth. In Fig. 2a the 20-hr. sample represented the beginning of logarithmic growth, and the 27-hr. sample its completion. In Fig. 2b logarithmic growth commenced at the 16th hour and had ceased by

the 30th. On the other hand it was not uncommon for the growth to begin almost at once, and the usual time required was about 8 hours. This variation will be made the subject of further study.

It was concluded that the buffer action of the medium was largely due to the ammonium sulfate it contained, and that the greatest buffer activity lay in the pH range near 9. This had a tendency to hold the pH high during early phases of growth, permitting it to drop into the optimum pH range at about the time logarithmic growth was at its height.

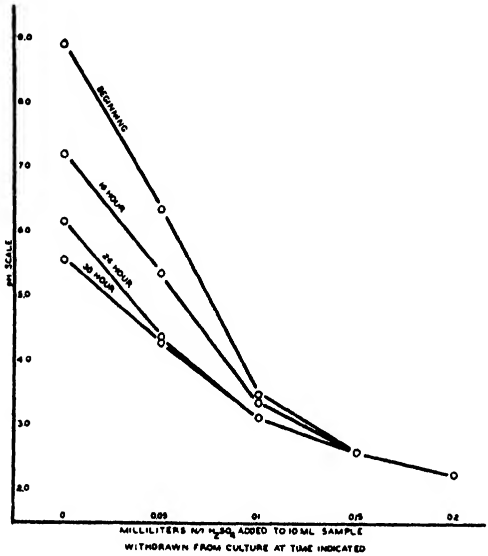

FIG. 2b

Lack of Buffering Capacity in Medium Containing Minimal Amount of Ammonium Sulfate Capable of Supporting Observable Growth

The medium contains 5 mg. of $(NH_4)_2SO_4$ per liter

The Influence of Aeration. The use of aeration, as advocated by Rahn (5) for other organisms, was resorted to in view of the observation that shallow cultures exceeded in final crop otherwise similar cultures with less relative exposed surface. Much better growth was obtained by this expedient, and it was felt that the actual influence of the bubbling should be ascertained. It seemed possible that the effect might be the purely mechanical one of stirring, or that some objectionable volatile substance might be removed, or that the increase in the liquid-air surface supplied the proper concentration of dissolved gases in the medium.

If the beneficial action was due entirely to the mechanical effect the use of nitrogen should serve as well as air. This did not prove to be the case. Some improvement in growth over that of a perfectly quiet culture did occur, but it was not sufficient to account for the results with air.

The inefficacy of nitrogen as a bubbling gas answered to a certain degree the problem of whether the removal of some volatile material was the chief function of the aeration. Nitrogen again should serve as well in this respect as air. Variable amounts of ammonia were released from the $(NH_4)_2SO_4$ at the high pH used, and analysis of the exhaust gases for acid and alkali could only be made by comparison with an uninoculated control. Failure to demonstrate acid or base in the gases under these conditions was not considered sufficient evidence for a denial of their existence in small quantities. The attempt, however, led to an interesting observation.

To measure the CO_2 which must be given off by the culture an experiment was set up in which air, washed free of CO_2 in concentrated KOH, was passed through the medium. It was found that when the air was so treated growth did not occur. It has frequently been shown (6) that CO_2 is necessary for the growth of certain microorganisms and it appears that such is the case with the cholera vibrio. It appears that a greater concentration of this substance than is normally present in the air is definitely desirable. The best results from the standpoint of obtaining a large crop have been obtained when the culture is either submitted to an initial period of bubbling with pure CO_2 or when the air used for aeration is mixed with 5% CO_2 throughout the growth period. Growth was commenced earlier and continued to a much higher total yield of vibrios than when CO_2 was not used.

The possibility of a CO_2-bicarbonate acid regulating system operating under these conditions cannot be overlooked, and very promising experiments along these lines are now under way, to be reported in a subsequent communication.

The conclusion at present must be that the bubbling serves to create the proper gas-liquid relationships rather than to act as a mere stirring device or as an excretory mechanism in the sense of washing objectionable gaseous material out of the culture.

Casein Digest as a Nutrient. The inclusion of casein digest in the medium came as a result of the desire to incorporate only such materials as would be easily removable by dialysis. As we have indicated (1) 0.2% peptone served as well under the conditions we have employed

as did the usual 2% peptone. The peptone, however, had the objectionable quality of imparting a color to the medium which could not be removed by dialysis, even though the peptone solution itself had been dialyzed through Visking tubing before use. Actually, the use of the small amount of peptone appeared to be more satisfactory than did the casein digest, possibly due to a larger content of growth-stimulating substances. However, the casein digest gives very acceptable results, and as stated is more suited for the subsequent treatment of the culture.

That some source of organic nitrogen was essential was demonstrated by failure to obtain any growth when neither casein digest nor peptone was incorporated in the salt medium.

Summary and Conclusions

The influence of various factors contributing to a maximum growth of *Vibrio cholerae* in the dialyzable salts-casein digest medium described in this series of communications has been evaluated as follows.

1. Glucose. Increasing concentrations of glucose, up to 0.3%, improve the final crop yield. When 0.3% sugar has been consumed, growth ceases because of the accumulation of acid.

2. pH. The best growth occurs within the range lying between pH 6.0 and pH 8.0. A high initial pH is desirable since it permits full use of this range before the accumulation of acid terminates growth. The total range which may be covered by a culture is from about 9.9 to 5.5. Higher or lower hydrogen ion concentrations than these prevent growth entirely.

3. Ammonium Sulfate. The buffering activity of the medium is largely due to the presence of $(NH_4)_2SO_4$. This material provides its greatest buffering action over a pH range near 9, thus restraining acidification of the medium until growth is well under way.

4. Casein Digest. A source of organic nitrogen and growth-stimulating substances is necessary but does not need to be as concentrated as in the classic media. Dialyzed peptone is more beneficial than a tryptic digest of casein but the latter is satisfactory and better suited for the subsequent manipulations in preparing vaccines, antigenic fractions, or bacterial polysaccharides.

5. Aeration. Aeration enhances growth by providing the proper concentration of dissolved gases rather than by serving as a stirring mechanism or as a means of removing objectionable volatile matter. An important factor is the presence of sufficient concentration of CO_2, which should be greater than the normal CO_2 content of air.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Biochemical Research Foundation of the Franklin Institute.

Note by the Director: The consideration of this contract was one dollar.

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Growth Requirements of the Photosynthetic Bacterium *Rhodospirillum rubrum*

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Introduction

The metabolism of the photosynthetic bacteria has been the subject of many recent investigations, from which have come a deeper insight into photosynthesis than has been derived from the study of algae and higher plants alone. These developments have been reviewed by Franck and Gaffron (1) and by Van Niel (2). Van Niel's paper provides a comprehensive critical summary of present knowledge of the metabolism of the photosynthetic bacteria. The non-sulfur purple bacteria (*Athiorhodaceae*), to which *Rhodospirillum rubrum* belongs, are of special value in elucidation of the connection between heterotrophic and autotrophic metabolism because of their ability to reduce CO_2 photochemically with a large number of organic compounds. Work with members of the *Athiorhodaceae* involves a complication not met in the other two families of photosynthetic bacteria: "... growth is dependent on the presence of small amounts of complex organic materials, such as yeast extract, which presumably furnish necessary organic growth factors" (2). An identification of the growth factors for *R. rubrum* is contained in the following description of its growth in a medium containing biotin, glutamate, and glucose as sole organic constituents.

Experimental

The strain used[1] was selected because it grew profusely in mass cultures under aerobic conditions, in contrast to the great majority of the *Athiorhodaceae* which are anaerobic, at least when freshly isolated (2).

[1] We are indebted for the culture to Professor L. D. Bushnell of the Kansas State College of Agriculture and Applied Science who informs us that it was probably obtained originally from the University of Wisconsin. It appears identical with *Spirillum rubrum* #277 of the American Type Culture Collection. These cultures probably all stem from one isolated by Esmarch in 1887 and maintained since on artificial media.

The stock cultures were maintained on glucose-peptone slants or on semi-solid agar. Growth conformed to the description in Bergey (3) except in respect to temperature: Bergey lists 37°C. as the optimum temperature. A characteristic feature of slant cultures was the colorless surface colonies accompanied by deep red growth at the interface between agar and glass. The cultures were incubated at room temperature (22–30°C.) at a distance of 4–5 feet from a 60 watt tungsten lamp. Above 30°C. growth was irregular, with inhibition becoming more pronounced at higher light intensities. The level of illumination selected, while low, allowed rapid growth, and did not appear to constitute a limiting factor. The organisms were strongly phototropic. Light from a "daylight" fluorescent lamp, which had been successfully used for the equally temperature-sensitive green flagellate *Euglena gracilis*, was unsatisfactory for *R. rubrum*, a finding explained by the preference of purple bacteria for light of longer wave length than that optimal for higher plants. Culture stored in the dark at 5–6°C. continued to grow at an appreciable rate.

Experimental media were distributed in 10 ml. amounts in 50 ml. Erlenmeyer flasks covered with 20 ml. beakers, and were sterilized by autoclaving for 15 minutes at 121°C. Each flask was inoculated with a drop of the growth from either (1) a previous experiment in which the nutrient under consideration was present in minimal concentration, or (2) from some of the slant growth suspended in a basal medium lacking the nutrient. In either case at least two serial subcultures were made for each experimental series. With this procedure no unaccountable carry-over phenomena were observed. The growth appeared capable of indefinite serial subculture in the simplest of the synthetic media: one with glutamate as sole amino acid. Substantially full growth was generally obtained within ten days.

Glucose was added aseptically. A 40 per cent solution was acidified to pH 2.8–3.2 with H_2SO_4 and treated with activated carbon in the amount of 5% of the weight of the sugar. The carbon was filtered off with the aid of Super-Cel, and the carbon treatment was repeated on the filtrate. Concentrated solutions of even the analytical grades of glucose have a yellow tinge which is completely removed by the carbon treatment. Solutions more concentrated than 50 per cent are not readily decolorized. The final solution was distributed among small flasks and sterilized by heating at 121°C. No caramelization occurred, and making allowance for evaporation, the same sugar solutions could be re-sterilized over and over without damage. The basal solution to which the glucose was added had sufficient buffer to render neutralization of the glucose unnecessary.

Results and Discussion

A medium permitting extremely heavy growth is shown in Table I.

Rôle of Glucose. Judging from the literature, glucose is a rather unusual substrate for purple bacteria. *Dl*-lactate, acetate, butyrate, and succinate were well utilized, as indicated by alkalinization of the medium (reaching pH 8.6 – >9.0) as well as by growth. In the latter respect, the organism behaved as a typical member of the *Athiorhodaceae*. However, growth with glucose was more rapid and much heavier. Mannitol,

sorbitol, sucrose, and glycerol were not utilized. Either glucose or an organic acid such as one of those mentioned above was necessary for growth. Growth was less with concentrations of glucose below 1%.

The superiority of glucose may rest on more than one factor: (1) Glucose media remain nearly neutral in reaction, the pH never falling below 6.5. (2) The hydrogen of glucose may be more available as donor for CO_2 reduction. (3) Glucose may be glycolyzed in the usual manner, thus furnishing additional energy, and the cleavage products may serve in addition as H-donors. (4) Glucose, as a result of aerobic or anaerobic processes, may furnish an abundance of CO_2. It is to be noted that carbonate was not added to any of the media. (5) An aerobic energy-yielding process may be present, either a direct oxidation as is found in some fungi, or the cleavage products may be resynthesized in a Pasteur

TABLE I

Medium for Rhodospirillum rubrum in Light

Ingredients per 100 ml.

K_2HPO_4	0.05 g.
$MgSO_4 \cdot 7H_2O$	0.02 g.
Gelatin hydrolyzate	0.5 g.
Glucose	2.0 g.
Biotin	0.2 g.
Fe 10 γ, Zn 5.0 γ, Ca 10 γ, Cu 1.0 γ, Mn 1.0 γ, Mo 1.0 γ, B 0.5 γ, I 0.1 γ	
H_2O to 100 ml.	
pH 7.4–7.6	

cycle. This organism is one of the most aerobic of the non-sulfur purple bacteria, yet the slight acidification of the medium is constantly present.

Amino Acid Requirements. Media containing gelatin hydrolyzate allowed the heaviest growth over a comparatively short period. Over a longer period, growth was almost as heavy with mixtures of amino acids. The hydrolyzate was prepared in the usual maner with H_2SO_4, and, after removal of most of the acid, the hydrolyzate was treated at pH 3.0 with carbon in the same manner as employed for glucose. These preparations gave no growth whatever without added biotin. The simplest amino acid mixture supporting heavy growth consisted of Na H *d*-glutamate 0.05%; *d*-arginine hydrochloride 0.03%; and cystine 0.02%. Glutamate was indispensable and could not be replaced by any other amino acid. While the amount needed cannot yet be stated in

quantitative terms, growth falls off below 0.05%. Glutamate appeared to be the primary source of nitrogen as ammonium salts were ineffective. As mentioned above, growth occurred in a simple glucose-biotin-glutamate medium, and although slow, it appeared indefinitely subculturable (at least 6 transfers). At each transfer, growth progressed steadily, becoming fairly heavy, until brought to an end by evaporation of the medium. Inclusion of arginine and cystine doubled the growth rate, and, by addition of several other amino acids, final growth was occasionally as heavy as with hydrolyzate media. An assessment of the value of the individual amino acids in these mixtures has not yet been carried out.

Biotin.[2] The indispensability of biotin was easily demonstrated after the need for the other nutrients had been established, using small amounts of urine or yeast extract as source of the then unidentified growth factor. The growth-promoting property of urine or yeast extract was increased somewhat by autoclaving with an excess of $Ba(OH)_2$; very small amounts of these alkali-treated materials sufficed for full growth. Treatment of urine with 5 per cent H_2O_2 at pH 8.0 for one hour at 100°C. led to inactivation. Activity was restored by biotin, and it was next found that biotin alone was needed. The response to biotin is shown in Fig. 1. Pimelic acid was inactive, as were the other usual growth factors.

Growth in Darkness. R. rubrum grows well in darkness at a rate roughly half that in light. At room temperature in liquid media, the growth is light pink; on agar in the refrigerator it is an intense wine-red. In darkness the superiority of hydrolyzate media is not manifest: growth in a mixture of glutamate and arginine is good and surpasses that in the hydrolyzate medium.

Comparative Position of Rhodospirillum rubrum as a Heterotrophic Organism. Burk and Winzler (4) have suggested that biotin may be the coenzyme for transfer of CO_2 since yeast grown with the diamino carboxylic acid derivative of biotin require more CO_2 for growth. Closure of the urea ring of biotin provides a mechanism for the uptake of CO_2. If *R. rubrum* were viewed as a unique example of an autotrophic organism assuming the heterotrophic mode of life it would be tempting to attach a special importance to the rôle of biotin in this transition. But organisms of unrelated groups undergoing an analogous change present an array of dissimilar patterns of nutrition. For example, some strains of *Chlorella* and other green algae can be grown in the dark with glucose

[2] We wish to express our gratitude to Merck and Co. for a generous gift of biotin.

as the sole organic nutrient (5). Again, the euglenoid flagellates cannot utilize any hexoses at all, metabolizing instead the lower saturated fatty acids containing an even number of carbon atoms (6). The green *Euglena gracilis* has been grown in the dark with acetate alone (7);

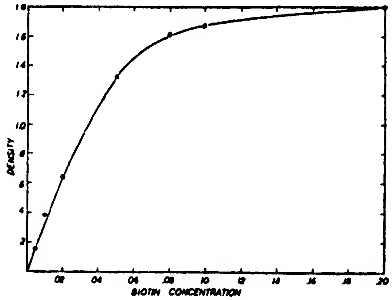

FIG. 1

Growth of *Rhodospirillum rubrum* as a Function of Biotin Concentration (expressed as γ per cent) in a Hydrolyzate Medium

The data are plotted as optical density against amount of biotin supplied. Since

$$\text{the optical density}\left(= \log \frac{\text{intensity of light transmitted through clear culture medium}}{\text{intensity of light transmitted through turbid culture medium}}\right)$$

was measured under conditions for which Beer's law is applicable, the density is directly proportional to the number of organisms per ml., and the straight-line character of the curve for low concentrations of biotin is significant, indicating that the total amount of growth is linearly proportional to the amount of biotin supplied.

Optical densities were measured with the precision photoelectric densitometer manufactured by the National Photocolor Corporation, New York. The instrument was slightly modified to measure the light absorbed or scattered from a narrow beam passing through a small rectangular cell in which the cultures were placed. The instrument reads directly in density units to a precision of ± 0.005. This characteristic makes it unnecessary to convert per cent transmission into optical density in order to obtain a reproducible physical quantity which is linearly proportional to the number of organisms.

also the colorless euglenoid *Astasia* (8). Inasmuch as all non-autotrophic organisms may be said to be derived from autotrophic ancestors, it is clear that many distinct paths have been followed in the development of heterotrophy.

Like most other Gram-negative bacteria, *R. rubrum* has an endotoxin producing a characteristic vascular damage when administered paren-

terally to animals (9). However, this immunochemical relation to the non-photosynthetic Gram-negative bacteria is not supported by a comparable nutritional resemblance, not even among the spirillae. Myers (10) isolated many spirillae and vibrios from soil and water, all of which grew with ammonium salts as the nitrogen source. Pennington (11) described a *Spirillum serpens* which required purines in an asparagin medium. There appears to be no report aside from the present study of a biotin requirement in any of the genera allied to *Spirillum* (*Vibrio*, *Pseudomonas*, *Protoaminobacter*).

Summary

The facultative photosynthetic non-sulfur bacterium *Rhodospirillum rubrum* has been grown in light and in darkness on a medium containing glucose, glutamate, and biotin as the sole organic constituents. The biotin requirement has been presented in quantitative terms.

The versatility of this organism, demonstrated by its ability either to live in light partly by the aid of photosynthesis, or to grow well in the dark without photosynthesis; its ability to grow aerobically, or under suitable conditions, anaerobically; and its utilization of either glucose or organic acids, indicate that this organism may be of special value in the study of the mechanisms of both light and dark metabolism.

A comparison of *R. rubrum* with other organisms undergoing an analogous transition from autotrophic to heterotrophic nutrition underlines the need for caution in attributing a specific rôle to biotin in this process.

The author is indebted to Dr. F. S. Cooper for demonstrating the application of the densitometer to the measurement of bacterial growth.

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Studies on the Assimilation of Dicarboxylic Acids by *Pseudomonas saccharophila*

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Introduction

Investigations on oxidative assimilation by Barker (1936), Giesberger (1936), Clifton (1937), Doudoroff (1940), and Pickett and Clifton (1943), have indicated that non-nitrogenous organic substrates are partially respired to carbon dioxide and water, and partially converted to cell material by suspensions of living cells. It has been found that the synthesis is of such constancy as to permit equational representation. For example, Clifton and Logan (1938), working with *E. coli*, by determining the respiratory quotient and assuming that the synthesized material was carbohydrate, represent the oxidative assimilation of fumaric, succinic, and pyruvic acids respectively as:

$$C_4H_4O_4 + 2\ O_2 \rightarrow (CH_2O) + 3\ CO_2 + H_2O \tag{1}$$

$$C_4H_6O_4 + 2\tfrac{1}{2}\ O_2 \rightarrow (CH_2O) + 3\ CO_2 + 2H_2O \tag{2}$$

$$2\ C_3H_4O_3 + 3\ O_2 \rightarrow 2\ (CH_2O) + 4\ CO_2 + 2H_2O \tag{3}$$

Doudoroff (1940), investigating the utilization of sugars and other organic compounds by the facultatively autotrophic "hydrogen bacterium" *Pseudomonas saccharophila* found that in the decomposition of most sugars, as well as of lactic and pyruvic acids, one third of the molecule was oxidized while two thirds were assimilated.

It seemed likely that a study of the utilization of the four-carbon dicarboxylic acids by the same organism would contribute further to an understanding of oxidative assimilation, especially since these compounds have assumed a place of particular interest to students of cellular physiology.

The experiments to be reported here deal with the oxidative assimilation of succinic, fumaric, malic, and oxalacetic acids by resting cells of *Ps. saccharophila* and are based chiefly on manometric methods. Some investigations on the effects of the selective poisons, 2,4-dinitro-phenol and malonic acid, will also be included.

Methods

The organism employed in these studies, *Ps. saccharophila* (Doudoroff), was grown in a synthetic medium containing distilled water, $M/30$ Sörenson KH_2PO_4–Na_2HPO_4 buffer at pH 6.46, 0.1% NH_4Cl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% $FeCl_3$, 0.005% $CaCl_2$, and either succinate or fumarate as carbon source in concentrations of 0.3%–0.5%. As a result of the utilization of the organic acid constituent, the pH value of the medium increased progressively with growth. The cultures were allowed to develop until a desired pH had been attained (to be referred to hereafter as "*growth pH*"). The bacteria were then centrifuged, resuspended in $M/20$ buffer at pH 6.64, and starved by aeration on a mechanical shaker to reduce autorespiration to a minimum. Aeration for a period of six hours was found to be sufficient to decrease the oxygen uptake of resting cells to such an extent that it was found unnecessary to run controls for autorespiration in all of the experiments to be reported, with the exception of those in which dinitrophenol was added to the suspensions. After starvation, the cells were again centrifuged and resuspended in $M/15$ phosphate buffer at a desired pH (to be hereafter referred to as "*experimental pH*"). All experiments were run at 20–22°C., employing the usual Warburg manometric technic. Each vessel contained a liquid volume of 2.3 cc. and was shaken at a rate of 100 cycles per minute.

Experimental

1. *Young Cultures*

Since all cultures were started at pH 6.46, a young culture will be defined here, as one in which the growth pH did not exceed 7.0. With such cultures it was found that after the addition of any of the substrates investigated, the rate of oxygen uptake rapidly increased to a maximum, and remained thus until the substrate had disappeared, as evidenced by a sharp drop in the rate and an approach to the rate of autorespiration (Fig. 1 (A)).

The total amount of oxygen consumed was found to be 1.04 moles of oxygen per mole of fumarate oxidized, as an average in 50 experiments. Respiratory quotient determinations gave an average value of 2.02, indicating the production of approximately 2 moles of carbon dioxide per mole of fumaric acid. Thus, half of the fumaric acid molecule appears to be oxidized to carbon dioxide, while the other half may be presumed

to be synthesized to cell material (possibly carbohydrate, having the rough empirical formula CH_2O), in accordance with the following equation:

$$C_4H_4O_4 + O_2 \rightarrow 2\ CO_2 + 2\ (CH_2O) \quad (4)$$

Experiments with *l*-malate indicate that the course of its oxidation was identical with that for fumarate, as may be seen from both Figs. 1 (A)

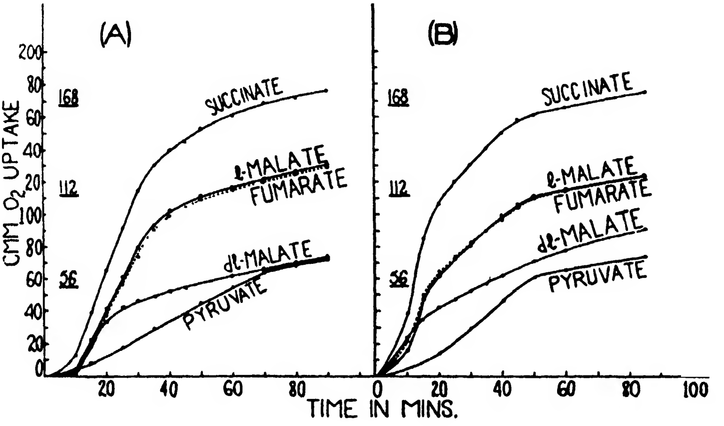

FIG. 1 (A) AND (B)

The Utilization of Organic Acids by *Ps. saccharophila*

The experiments were run at an experimental pH of 5.9 with 0.005 milliequivalents of substrate employed. (A) Curves for oxygen uptake by "young cultures". (B) Curves for oxygen uptake by "old cultures". (Note: 0.005 milliequivalents O_2 = 112 cmm.)

and 1 (B). Hence an equation for the oxidative assimilation of *l*-malate may be represented as:

$$C_4H_6O_5 + O_2 \rightarrow 2CO_2 + 2\ (CH_2O) + H_2O \quad (5)$$

The demonstration of identical rates for the utilization of fumarate and *l*-malate appears to be in agreement with the conclusion of Green (1936), that the so-called fumaric dehydrogenase is really the malic dehydrogenase collaborating with fumarase.

It may be seen from Fig. 1 that *dl*-malate is oxidized only to half the extent of the *l*-form, supporting the view that malic dehydrogenase is specific for *l*(-) malate (Green, 1936).

In a series of 30 experiments with succinate, the average total oxygen consumption was found to be 1.48 moles per mole of succinic acid. Hence the utilization of succinate may be represented as:

$$C_4H_6O_4 + 1\tfrac{1}{2}\ O_2 \rightarrow 2\ CO_2 + 2\ (CH_2O) + H_2O \qquad (6)$$

The fact that the rate of succinate utilization is almost identical with that of fumarate and malate, and that the amount of synthesis is almost the same from the three compounds, supports the view of Quastel and Wheatly (1931) that fumaric acid is an intermediate in the biological oxidation of succinic acid.

In experiments on the utilization of pyruvate by *Ps. saccharophila* approximately the same results were obtained as those described by Doudoroff (1940), namely, an average oxygen uptake of 0.59 moles per mole of substrate. He assumed that autorespiration might not be completely inhibited during the oxidation of pyruvate as it appears to be during the oxidation of other compounds. Thus the actual oxygen uptake for the isolated reaction might be closer to 0.5 moles, in accordance with the equation:

$$C_3H_4O_3 + \tfrac{1}{2}\ O_2 \rightarrow CO_2 + 2\ (CH_2O) \qquad (7)$$

2. Old Cultures

An "old culture" will be defined as one which had developed until the liberation of sodium ion, through the oxidation of sodium fumarate, had caused the medium to attain pH 8 or higher.

The course of oxidation of the dicarboxylic acids by such old cultures was found to be strikingly different from that observed with young cultures under similar conditions; compare Fig. 1 (A) and 1 (B). Instead of a single constant rate of oxygen uptake, two separate rates were observed, namely, a high initial rate and a lower subsequent one. This phenomenon did not appear with pyruvic acid as substrate.

The first rate continued until about 0.5 moles of oxygen had been used per mole of fumarate or *l*-malate, and 1.0 mole of oxygen was used per mole of succinate. A second rate was then established and remained constant until an additional 0.5 mole of oxygen had been used for any of the three acids. The total amount of oxygen consumed during the two stages of respiration was found to closely approximate the value obtained with young cultures, and about the same amount of carbon dioxide was found to be evolved in the over-all process. These observations coupled

with determinations of the respiratory quotients, suggested the accumulation and subsequent oxidation of an intermediate product of metabolism having an empirical formula approximating that of pyruvic acid ($C_3H_4O_3$). The appearance and later disappearance of a keto acid was indeed demonstrated in such experiments. This acid was identified as pyruvic acid by the preparation of its 2,4-dinitro-phenylhydrazone. (M.P. uncorrected of 2,4-dinitro-phenylhydrazone of pyruvic acid: 212.6°C.; unknown: 212.6°C.; mixed: 212.2°C.). It should be noted that Beijerinck (1916) has described the accumulation of pyruvic acid during the utilization of malic acid by a number of bacteria.

That the utilization of the accumulated pyruvic acid in the medium accounted for most of the oxygen uptake during the second period of respiration, could be shown by a simple experiment. The bacteria were separated from the medium by centrifugation immediately after the termination of the first period, that is, after the first decrease in rate of

TABLE I

Experiment Showing the Extracellular Accumulation of Pyruvic Acid during the Second Rate

	cmm. O_2 uptake *per 30 minutes*
(1) Bacteria centrifuged after first period of respiration of fumarate, resuspended in their own supernatant.	20
(2) Bacteria as in (1), but resuspended in buffer.	5
(3) Starved bacteria in buffer.	2
(4) Starved bacteria in supernatant from (1).	19

oxygen consumption. These bacteria were resuspended in phosphate buffer, while starved bacteria were suspended in the supernatant. As will be seen from Table I, the substrate responsible for the second rate was found to be almost entirely in the medium.

An interesting observation was made on the effect of iron concentration of the medium on the physiological "ageing" of the cultures. If insufficient iron is added (0.0005% $FeCl_3$), the cultures do not develop to as great an extent as those with higher iron concentrations, and the pH of the medium does not rise to as high a value. Cultures grown in such iron-deficient media behaved like "young cultures" towards the dicarboxylic acids, even though left in fairly alkaline media for long periods of time before harvesting. Resuspension, and prolonged aeration, of such cultures in buffer at pH 8.0 did not change their behavior. The addition of iron salts to the bacterial suspension in respirometer experi-

ments was also without effect. It would appear from these observations that the change from "young culture" to "old culture" course of metabolism, is associated with the proliferation of the bacteria in the altered alkaline conditions. The iron deficiency either prevents the proliferation, or in some other way prevents the transition from one type of oxidation to the other.

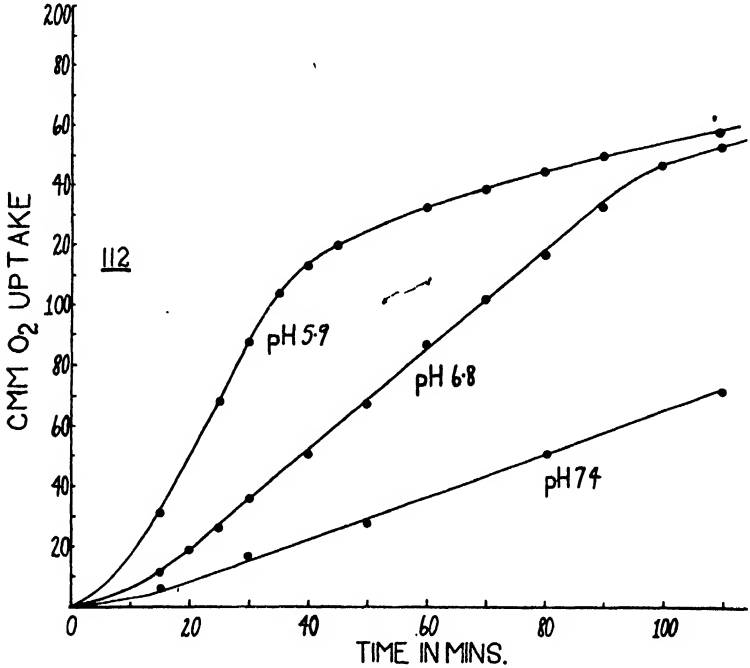

Fig. 2

Oxidation of Fumarate at Different Experimental pH values by a "Young Culture"

Bacteria harvested from culture at pH 6.8, suspended in $M/15$ buffer. 0.005 meq. fumarate used as substrate. (Note: 0.005 meq. O_2 = 112 cmm.)

The changes in the course of dicarboxylic acid utilization accompanying the ageing of the cultures could be demonstrated clearly only if the experimental pH, that is, the pH of the suspension in the respirometer, was kept low (pH 5.9 − 6.2). Under more alkaline conditions, the rate of oxidation of these substrates was reduced (Figs. 2 and 3).

It will be noted from Fig. 3 that the division of the metabolism of old

cultures into two phases is obscured at higher pH values. That this is due to a decrease in the rate of oxidation of fumarate, unaccompanied by a similar decrease in that for pyruvate, may be judged from the results of experiments in which the substrates were supplied at different pH values (Tables II and III).

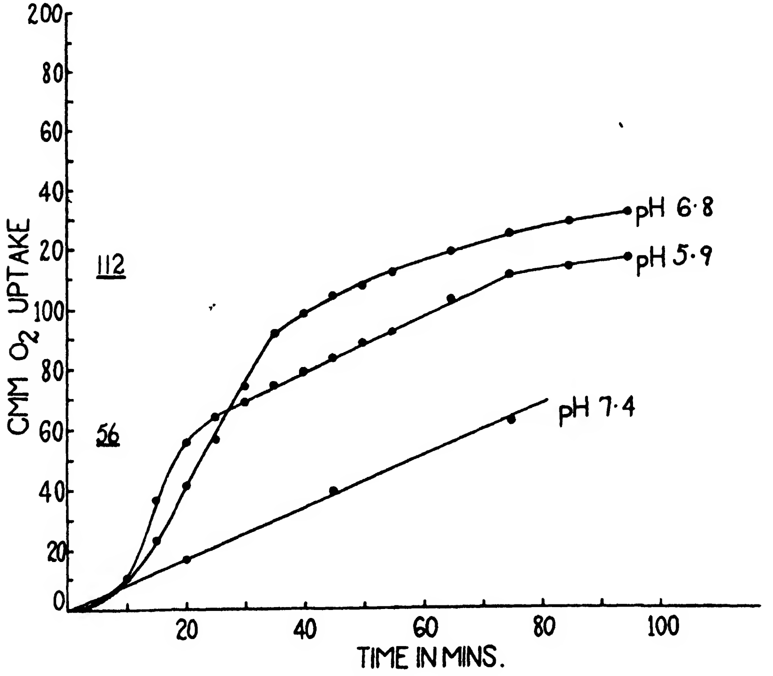

FIG. 3

Oxidation of Fumarate at Different Experimental pH Values by an "Old Culture" Bacteria harvested from culture at pH 8.0 suspended in $M/15$ buffer. 0.005 meq. fumarate used as substrate. (Note: 0.005 meq. O_2 = 112 cmm.)

It will be noted from Fig. 1 (A) and Table II that the rate of pyruvate utilization at pH 5.9 by young cultures is insufficient to account for the "single phase" oxidation of fumarate, if pyruvic acid is assumed to be an intermediate in the metabolism of such cells. No pyruvic acid could ever be detected in the medium in the course of oxidation of fumarate by young cultures.

However, in view of the similarity of the over-all process carried out by young and old cultures, it would seem unlikely that pyruvic acid is an

intermediate only in the metabolism of the latter. It therefore appears probable that pyruvic acid occurs also in the metabolism of fumaric acid by young cells, but can be used more rapidly when produced intracellularly than when supplied from the outside. A higher rate of its utilization could be due to its appearance in the phosphorylated form, its immediate condensation with some other compound, its proximity to the enzyme systems, or the catalytic effect of fumarate.

TABLE II

Rates of Oxygen Consumption by Suspensions of Ps. saccharophila at Different Experimental pH's for (A) a Young Culture, (B) an Old Culture

Numbers Represent cmm. O_2 Uptake per 10 mins.

(A) Young Culture Substrate (0.005 meq.)	Experimental pH 5.9	Experimental pH 7.4	(B) Old Culture Substrate (0.005 meq.)		Experimental pH 5.9	Experimental pH 7.4
Fumarate	34	5	Fumarate	1st rate	59	16
				2nd rate	11	
Pyruvate	9	10	Pyruvate		11	12

TABLE III

Q_{O_2}*'s for Different Aged Cultures of Ps. saccharophila, Respiring 0.005 meq. Fumarate, at Different Experimental pH's*

Growth pH		Q_{O_2} at Experimental pH 5.9	6.8	7.4
6.8		93.5	39.5	15.6
7.6	1st rate	29.4	19.4	10.3
	2nd rate	7.7		
8.3	1st rate	10.3	6.8	2.4
	2nd rate	2.0		

3. The Utilization of Oxalacetic Acid

Oxalacetic acid was prepared according to the method of Krampitz and Werkman (1941). The purity of the resulting compound, crystallized from acetone-chloroform mixture, varied between 80–100%, as determined by the aniline method of Ostern (1933). A solution of the sodium salt adjusted to the desired pH was used in the experiments. Controls for spontaneous decarboxylation were included in all experiments.

Since pyruvic acid appears to be an intermediate in the oxidation of fumaric and malic acids, it seems reasonable to assume that it arises through the decarboxylation of oxalacetic acid as follows:

$$\text{Malic acid} + \tfrac{1}{2}\,O_2 \rightarrow \text{Oxalacetic acid} + H_2O \tag{8}$$

$$\text{Oxalacetic acid} \rightarrow \text{Pyruvic acid} + CO_2 \tag{9}$$

Equation 9 would indicate that oxalacetate should be decarboxylated anaerobically, by this obligately aerobic *Pseudomonas*. In a series of four experiments, no anaerobic decarboxylation could be detected manometrically, and determinations made on the contents of the Warburg vessels at the conclusion of the experiments showed no disappearance of oxalacetic acid.

Aerobically, oxalacetic acid was oxidized readily, and at a rate comparable to that for fumaric or malic acids. From the study of the utilization of the latter compounds, one would expect one atom of oxygen uptake and two molecules of carbon dioxide produced for each molecule of oxalacetate oxidized. However, it was found that although approximately the expected amount of carbon dioxide was liberated, twice the theoretical amount of oxygen was utilized. This observation was made with both young and old cultures. The above result indicates the formation of an unidentified product, considerably more oxidized than carbohydrate, either in the bacteria or in the medium. No pyruvic acid could be detected in the medium after the oxalacetic acid had disappeared.

4. The Effect of 2,4-dinitro-phenol on the Oxidative Assimilation of Dicarboxylic Acids

Several investigators have shown that suitable concentrations of 2,4-dinitro-phenol (hereafter *referred to as* DNP) increases the rate and extent of oxidation of substrates by resting cells. Clifton (1937), Clifton and Logan (1938, 1939), and Doudoroff (1940), from experiments with *Ps. calco-acetica*, *E. coli*, and *Ps. saccharophila*, respectively, concluded that DNP interferes with the assimilatory, but not with the catabolic mechanism. Recently Burris and Wilson (1942) have shown that in the oxidation of glucose by *R. trifolii* 205, it is unnecessary to postulate a primary inhibition of assimilation to explain increased substrate oxidation, since the same effect was observed with DNP added initially, or immediately after assimilation of glucose had been completed.

It is of interest to note that Doudoroff (1940) with *Ps. saccharophila*, has shown complete oxidation when sugars were employed as the substrates. However, in the present studies with the same organism, fumaric and succinic acids were never oxidized to completion in the presence of this poison. Concentrations of DNP from $M/2000$ to $M/100{,}000$ were employed at pH 5.9. Optimal results were obtained with $M/40{,}000$ DNP, when the substrate was used at a concentration of $M/800$. It has been assumed that in the presence of DNP autorespiration continues unaffected by the addition of substrate (Doudoroff, 1940). Hence, for all experiments, autorespiration values obtained with cells respiring in the presence of DNP without substrate, were subtracted from the values observed for oxygen consumption and carbon dioxide production in the presence of the substrate.

Great difficulty was encountered in defining the point of substrate exhaustion. At no time during the utilization of either fumaric or succinic acids would subtraction of the control autorespiration give theoretically expected results for complete oxidation of the substrate. The respiratory quotient (R.Q.) was always somewhat high in the initial stages of oxidation (*e.g.* 1.46 for fumarate; theoretical 1.33) and later approached the expected theoretical R.Q. for complete oxidation. The initial higher R.Q. indicated the accumulation of some intermediate compound during the early stages of oxidation. This compound was found to be pyruvic acid, which eventually disappeared as the oxidation proceeded. The same observation was made when succinate was employed as the substrate.

In all cases, the presence of DNP definitely resulted in increased oxidation of the dicarboxylic acid substrate, but never in what could be unequivocally interpreted as complete oxidation. This would indicate either that the corrections made for autorespiration were unwarranted in the present experiments, or that synthetic processes are not completely suppressed by DNP with the substrates employed.

5. The Effect of Malonate on the Oxidation of Various Organic Acids

The enzymatic oxidation of succinic acid is strongly inhibited by low concentrations of malonic acid (Quastel and Wheatley, 1931), and can be completely abolished by high concentrations. In the presence of high concentrations of this poison other dehydrogenases may possibly be affected as well as succinic dehydrogenase (Das, 1937).

The rate of oxidation of succinic, fumaric, and pyruvic acids by *Ps.*

saccharophila was decreased, but only when malonic acid was present in relatively high concentrations. A typical experiment with malonate is shown in Table IV.

At the end of one hour, $M/20$ malonate only inhibited the oxidation of succinate by 71%, of fumarate by 59%, that of pyruvate by 27%. $M/1000$ malonate apparently exerts no inhibitory action whatever.

The concentrations of malonate causing inhibition are thus of an entirely different order from those used by Krebs (1940), who found that the action of succinic dehydrogenase is strongly affected by $M/1000$ malonate. It is impossible to decide whether the explanation lies in the very poor penetration of malonate into the cells, or in the insensitivity of the bacterial enzymes to this poison.

The addition of $M/100$ malonate, to either $M/400$ succinate or fumarate, had no significant effect on the assimilatory reactions as evidenced by the unchanged total oxygen consumption. However, the rate of oxygen utilization was definitely decreased in the presence of this poison.

TABLE IV

The Effect of Various Concentrations of Malonate on the Oxidation of M/40 Succinate, Fumarate, and Pyruvate by Ps. saccharophila

(The figures indicate cmm. O_2 uptake.)

Conc. of Malonate	*M/40 succinate*		*M/40 fumarate*		*M/40 pyruvate*	
	30 mins.	*60 mins.*	*30 mins.*	*60 mins.*	*30 mins.*	*60 mins.*
$M/20$	17	48	13	48	20	47
$M/40$	33	83	22	74	24	56
$M/100$	50	123	38	97	31	67
$M/1000$	75	169	46	116	27	63
0	72	163	46	116	28	64

Discussion

The present investigations have shown clearly the danger of relying on rates or changes in rates of oxygen consumption alone, in interpreting the course of oxidation or assimilation of substrates. Very marked differences in the course of oxidation may be rapidly induced by changes in cultural conditions resulting from the growth of the bacteria, and may depend on some factor not obviously connected with such changes (*e.g.* concentration of iron and pH of the medium).

The assimilatory mechanism appears to be fairly insensitive to the factors influencing the course and rate of oxidation, as evidence by the "ageing" of the cells.

The observations on the rates of substrate oxidation and the demonstration of the accumulation of pyruvic acid under special conditions, would lead to the postulation of the following scheme for the respiration of dicarboxylic acids:

1. Succinic acid + $\frac{1}{2}$ O_2 → Fumaric acid + H_2O
2. Fumaric acid + H_2O → *l*-Malic acid
3. *l*-Malic acid + $\frac{1}{2}$ O_2 → Oxalacetic acid + H_2O
4. Oxalacetic acid → Pyruvic acid + CO_2
5. Pyruvic acid + $\frac{1}{2}$ O_2 → CO_2 + 2 (CH_2O) (cell material)

At present only the manner of breakdown of oxalacetic acid does not conform to the above scheme. The elucidation of the mechanism of the decomposition of this compound may throw light on the reactions involved in both the catabolic and the primary anabolic processes.

It must be remembered that the proposed equation for the synthesis of cell material from pyruvic acid (step 5) is intended only as an empirical expression of the observed results. The actual process may well involve the complete oxidation of one molecule of pyruvic acid coupled with the reduction and synthesis of two others. It is interesting to note that a slightly greater amount of synthesis appears to occur when succinic or fumaric acids are used as substrates, even in cases where pyruvic acid accumulates in the course of the oxidation, than when the latter compound alone is supplied to the organism. This would seem to indicate that a small amount of energy for synthetic processes may be derived from oxidations preceding the formation of pyruvic acid.

The striking influence of pH, not only on the rate, but also on the course of metabolism of the dicarboxylic acids suggests either that these compounds penetrate the cell membrane very poorly at high pH values, or that some enzyme loses its activity under these conditions.

From the comparison of the effect of DNP on assimilation observed by Doudoroff (1940), with that found in the present studies, it would appear that this poison is less effective when dicarboxylic acids are used, as substrates, than when sugars, pyruvic, or lactic acids are utilized. It is of interest to note that in experiments with acetic acid, Doudoroff[1] has encountered the same difficulties in interpretating the effect of DNP and of suppressing synthesis as reported here. The apparent differences in the degree of inhibition of assimilation by DNP, depending on the choice of substrate, make it possible to hope that the mode of action of this poison may eventually be clarified.

[1] Personal communication.

I wish to express my deep gratitude to Dr. M. Doudoroff, of the Department of Bacteriology, and to Dr. H. A. Barker, of the Division of Plant Nutrition, for their generous advice and assistance in these investigations.

Summary

1. In the utilization of succinic, fumaric, and *l*-malic acids by "young cultures" of *Ps. saccharophila*, one half of the molecule is assimilated and one half completely oxidized. Fumaric and *l*-malic acids are used at the same rate.

2. "Old cultures" of *Ps. saccharophila*, although quantitatively assimilating the same fraction of the substrate as "young cultures," show two distinct stages in the course of oxidation which cannot be demonstrated with "young cultures." Pyruvic acid accumulates as an extracellular intermediate product in the first phase and disappears in the second.

3. pH exerts a remarkable influence on the rate and course of utilization of fumaric, succinic, and *l*-malic acids. The enzymatic stages demonstrated for "old cultures" occur only when the substrate is supplied to cell suspensions at relatively low pH values. In neutral or slightly alkaline buffers, the Q_{O_2} decreases, and the two phases appear to be concurrent.

4. The oxidation of oxalacetic acid by *Ps. saccharophila* does not seem to fit a simple scheme proposed for the oxidation of four carbon dicarboxylic acids. This substrate was not decarboxylated anaerobically by intact cells.

5. Although poisoning with 2,4-dinitro-phenol considerably decreases the extent of assimilation with dicarboxylic acids, the inhibition is less marked than with other substrates, and the experimental results are difficult to interpret.

6. Unusually high concentrations of malonate are required for the effective inhibition of succinic acid oxidation. At these concentrations the oxidation of fumaric acid is markedly inhibited and that of pyruvic acid to a lesser extent. The synthetic mechanism appears to be relatively insensitive to malonate inhibition.

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Studies on the Nutritional Requirements of *Clostridium thermosaccharolyticum*

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Introduction

It has been observed that *Clostridium thermosaccharolyticum* grows readily in liver infusion medium but fails to grow in many other anaerobic media unless large numbers of cells are used as an inoculum. The question was raised as to whether these media might be lacking in some metabolites essential for growth. Since these requirements were not known the object of this study was their determination. Interest in this organism results from the fact that it is important in food spoilage.

In recent publications the metabolite requirements of some of the mesophilic anaerobes have been presented. So far as we know this is the first report on the requirements of thermophilic anaerobes.

Since this work requires a synthetic or semi-synthetic medium various compositions were investigated. It was found that a medium having the following composition supported growth and yielded maximum acid production by the strain of *Clostridium thermosaccharolyticum* used in this work.

Composition of Medium per 100 ml.

Vitamin-free hydrolyzed casein	.5	g.
Cystine	10.0	mg.
Glucose	2.0	g.
Sodium acetate	.6	g.
Uracil	1.0	mg.
Inorganic salt mixture		
Solution A	.5	ml.
Solution B	.5	ml.
Thiamine hydrochloride	10.0	γ
Biotin	.01	γ
p-Aminobenzoic acid	.2	γ
Nicotinic acid	10.0	γ
Calcium pantothenate	10.0	γ

Reaction adjusted to pH 6.6–6.8

Solution A consists of a mixture of 10% K_2HPO_4 and 10% KH_2PO_4 in distilled water.

Solution B consists of a mixture of the following salts; 4% $MgSO_4 \cdot 7H_2O$; 0.2% NaCl; 0.2% $FeSO_4 \cdot 7H_2O$; 0.2% $MnSO_4 \cdot 4H_2O$ in distilled water.

For use, this medium was distributed in 10 ml. amounts into tubes and a nail reduced with concentrated HCl, carefully washed with distilled water was added just prior to sterilization to produce anaerobic conditions.

Methods

Strain No. 3814 of *Clostridium thermosaccharolyticum* chosen for this work was obtained from the National Canners Association. A similar culture was used by McClung (1935) in characterizing and naming this organism. Stock cultures were kept in liver infusion medium plus liver particles with $CaCO_3$ added to neutralize the acidity produced by the culture. From this stock a culture for experimental work was obtained by transferring to hydrolyzed casein medium. Serial transfers were made in this medium. Inocula were prepared by centrifuging a 48 hour culture, washing, then suspending the cells in 99 ml. of physiological saline. Two drops of this suspension was used as an inoculum for each tube.

Two types of anaerobic methods were used, namely stratification with mineral oil, and reduced iron as described above. Since the later method was more convenient for titration it was used in the work reported.

Glassware and test tubes were carefully cleaned with a solution of sulfuric acid and dichromate. Experimental media was sterilized in the autoclave at 121°C. for 15 minutes. In preliminary experiments the various metabolites were sterilized separately and added aseptically to the sterile medium. Results using this technic did not differ from those obtained when these substances were added to the medium previous to sterilization. Consequently metabolites were sterilized with the medium. *Clostridium thermosaccharolyticum* produced a measurable acidity in this medium. Results were reported as ml. of $N/10$ acid measured by $N/10$ NaOH produced in 10 ml. of the culture medium. The effect of the various metabolites was judged on the basis of increased or decreased acidity. Cultures were incubated for 72 hours at 55°C.

The nitrogen source used was vitamin-free hydrolyzed casein, and the usual difficulties in preparing a vitamin-free hydrolyzed casein were encountered. Previous to hydrolysis, the casein was extracted several times with 0.1 N acetic acid, then with 95 per cent ethyl alcohol. Hydrolysis was accomplished with 25 per cent H_2SO_4 and neutralized with $Ba(OH)_2$. The hydrolyzed casein was absorbed with norite at pH 3.0. The above procedure removed some of the hydrolyzed casein, the final product containing 50% of the original solids. Consequently the amount added to the medium was based on the concentration of total solids in the final solution. Before use, the hydrolyzate was tested for the absence of metabolites; riboflavin, pyridoxin, pantothenic acid, biotin, and folic acid by the assay procedure of Landy and Dicken (1942) using *Lactobacillus casei*; nicotinic acid by the method of Snell and Wright (1941) using *Lactobacillus arabinosus*; *p*-aminobenzoic acid by the method of Landy and Dicken (1942) using *Acetobacter suboxydans*.

Ammonium sulfate in the amounts tested, with or without asparagine could not replace hydrolyzed casein. The ammonium sulfate was added in three concentrations namely, 0.1%, 0.3%, and 0.5%; asparagine in 0.1% amount. Cystine and tryptophan were added as nitrogen sources in addition to the hydrolyzed casein. Omission of tryptophan did not result in decreased acid production which would indicate that tryptophan is not needed by the organism, however the hydrolyzed casein may contain sufficient of this amino acid to satisfy its requirements. When cystine was omitted a decrease in acidity resulted, hence it was considered necessary.

The purine bases adenine, guanine, uracil, and xanthine were added to the medium singly and in combination. When they were left out, definite decrease in acid production resulted. Uracil alone was as satisfactory as any combination of these substances. Guanine gave some stimulation but could not replace uracil. The addition of guanine to a medium containing uracil however gave no more response than uracil alone. Adenine and xanthine could be omitted from the medium since they gave no stimulation when added.

Stokes and Martin (1943) and Krehl, Strong and Elvehjem (1943) have shown that the ratio of sodium acetate to glucose influences acid production by *Lactobacillus casei* and *Lactobacillus arabinosus*. In an attempt to increase acid production by *Clostridium thermosaccharolyticum* varying ratios of sodium acetate and glucose were tried. A definite decrease resulted when sodium acetate was omitted from the medium, and there was decreased acid production when the amount of glucose was decreased. Doubling the amount of acetate did not produce marked increase in acid production. With normal amount of acetate the sugar concentration was found to influence acid production. The greatest increase was obtained when the sugar concentration was doubled. With greater amounts of sugar and sodium acetate, carmelization occurred and the titration of the uninoculated medium gave too high a blank.

The inorganic constituents were added as solution A containing the two phosphate salts, and solution B containing $MgSO_4$, NaCl, $FeSO_4$, and $MnSO_4$. The individual salts in each mixture were not tested. When both solution A and solution B were left out of the medium, very poor growth and acid production resulted. It appeared that both solutions were necessary for normal acid production. However, solution B could be added in half the amount suggested in the original formula without decreasing the acid production.

Metabolite Requirements

In the basal medium containing no metabolites, growth was uniformly poor with only slight acid production. Without attempting to present all the experimental data, it was observed that when thiamine, biotin, or *p*-aminobenzoic acid were absent from the medium, maximum acid production was not obtained. Table I indicates results using these three metabolites.

After the necessity for these three metabolites had been established the optimum amount of each was determined. Thiamine was first tested

by adding amounts varying from 1 γ/per cent to as high as 1 mg./per cent. Maximum acid production was obtained when 10 γ/per cent was added. With less than this amount, acid production decreased. Greater amounts neither increased nor decreased acid production. Using the above established amount of thiamine in the medium, biotin concentration of .01 γ/per cent gave maximum acid production. When the concentration of this metabolite was increased to 0.1 γ/per cent, slight inhibition resulted and marked inhibition occurred when 1.0 γ/per cent was added. *p*-Aminobenzoic acid in a concentration of 0.2 γ/per cent gave maximum acid production; 10 γ/per cent caused a decrease in titration; 100 γ/per cent gave a definite inhibition in both growth and acid production.

TABLE I

Response of Clostridium thermosaccharolyticum to Thiamine, Biotin, and p-Aminobenzoic acid

Medium	Titration *ml. N/10 NaOH*
Base only	.35
Base plus B_1*	.45
Base plus biotin	1.15
Base plus P.A.B.**	.40
Base plus B_1 and biotin	1.25
Base plus B_1 and P.A.B.	.60
Base plus B_1, biotin, P.A.B.	7.90

* B_1 = Thiamine.
** P.A.B. = *p*-Aminobenzoic acid.

After the requirements of *Clostridium thermosaccharolyticum* for these three substances had been determined other metabolites were tested. Pyridoxin, riboflavin, and folic acid gave no stimulation with the hydrolyzed casein used in the presence of these three metabolites. The addition of nicotinic acid and calcium pantothenate gave no stimulation in the original tests. After the organism had been cultivated for three months in medium with thiamine, biotin, and *p*-aminobenzoic acid slight stimulation resulted from the addition of nicotinic acid and calcium pantothenate. However continuous transfer of the organism can be made in media free of these two metabolites.

In order to further check the possibility of other stimulatory substances, media were prepared containing thiamine, biotin and *p*-aminobenzoic acid with and without nicotinic acid and calcium pantothenate.

To these media yeast extract was added to determine if this substance carried stimulatory substances other than those previously determined. Results are reported in Table II.

Addition of yeast extract to a medium containing thiamine, biotin, and *p*-aminobenzoic acid gave some added stimulation in acid production. When nicotinic acid or calcium pantothenate were included the addition of yeast extract yielded no further stimulation.

TABLE II

Effect of Yeast Extract, Nicotinic Acid, and Calcium Pantothenate on Acid Production in a Hydrolyzed Casein Medium Containing Thiamine, Biotin, and p-Aminobenzoic Acid

Medium	Titration *ml. N/10 NaOH*
Base only (uninoculated)	.45
Base only	1.35
Base plus biotin and P.A.B.	1.30
Base plus B_1 and biotin	5.80*
Base plus B_1, biotin and P.A.B.	7.90
Base plus B_1, biotin, P.A.B., and Yeast Extract	8.25
Base plus B_1, biotin, P.A.B., and NA**	8.30
Base plus B_1, biotin, P.A.B., and PA***	8.15
Base plus B_1, biotin, P.A.B., NA, and PA	8.25
Base plus B_1, biotin, P.A.B., NA, PA, and Yeast Extract	8.25

* It was found that this hydrolyzed casein had a small amount of *p*-aminobenzoic acid but not sufficient for maximum acid production.

Nicotinic acid and calcium pantothenate were added in a concentration of 10 γ/per cent. Larger amounts gave no further stimulation.

Yeast extract was added at rate of 0.6%.

** NA = Nicotinic acid.

*** PA = Calcium pantothenate.

As thiamine is essential for acid production by the test organism, the two fractions of this molecule were tested. Tests were run to determine the activity of 2-methyl-5-ethoxymethyl-6-amino-pyrimidine*, 4-methyl-5-β'-hydroxyethyl-thiazole*, and cocarboxylase.[1] These substances were added in molecular equivalent amounts based on thiamine. Table III gives the results.

The medium used in the above work was a complete hydrolyzed casein

[1] We wish to thank Merck and Company for supplying the pyrimidine, the thiazole, and cocarboxylase used in this work.

medium with thiamine omitted. The designations of γ in each column represents γ of thiamine or the equivalent amounts of these other test compounds.

Cocarboxylase was as active mole for mole as thiamine. The thiazole has thiamine activity. However, the organism required about ten times as much of this compound as thiamine to produce maximum acidity. The pyrimidine used in this work was not utilized. Only the one pyrimidine structure was used in these tests. When the thiazole and the pyrimidine were added in amounts equivalent to thiamine they were no more effective than the thiazole alone.

It has been shown by Du Vigneaud, *et al.* (1942) and Eakin and Eakin (1942) that pimelic acid may serve as a percursor in the synthesis of biotin for certain organisms. Since *Clostridium thermosaccharolyticum*

TABLE III

Response of Clostridium thermosaccharolyticum to the Pyrimidine, the Thiazole, and Cocarboxylase

Addition to medium	Titration in *ml.* *N/10 NaOH* (0.1γ) .0000000297 *mol.*	(1.0γ) .000000297 *mol.*	(10.0γ) .00000297 *mol.*	(100.0 γ) .0000297 *mol.*
Thiamine	5.75	8.50	8.40	8.30
Pyrimidine-compound	.70	.70	.70	.70
Thiazole-compound	3.35	5.80	8.35	8.35
Pyrimidine- and Thiazole-compound	3.30	4.65	8.40	8.40
Cocarboxylase	5.50	8.60	8.40	8.40

requires biotin, pimelic acid was substituted for biotin in the medium. Four concentrations were used namely .01, 0.1, 1.0, and 10.0 γ per 10 ml. of medium. The highest titration, 2.0 ml. of $N/10$ NaOH was obtained when 1.0 γ was used. Compared with previous results this low titration leads to the conclusion that, under the conditions of this experiment, pimelic acid cannot replace biotin in the nutrition of this organism.

Requirements of Other Strains of Thermophilic Anaerobes

In order to determine how other cultures of thermophilic anaerobes compared in their metabolite requirements with the National Canners Association strain used in this work, cultures were isolated from soybean flour and starch. The original culture from the National Canners Association which had been carried in liver infusion broth also was checked with the stock carried in hydrolyzed casein medium.

Because of the difficulty involved in preparing inoculum for this test from liver infusion medium with particles, all cultures were transferred to hydrolyzed casein medium. A second transfer was made in order to eliminate liver particles carried over from the original medium. Inoculum from each was prepared in the usual manner. Table IV indicates the results obtained.

Two cultures of thermophilic anaerobes isolated from soybean flour and from starch, as well as the N.C.A. No. 3814 stock strain carried in liver infusion medium showed a definite response to thiamine, biotin, and *p*-aminobenzoic acid, which is comparable to the results obtained

TABLE IV

Metabolite Requirements of Other Strains of Thermophilic Anaerobes Compared with N.C.A. No. 3814 Grown Continuously in Hydrolyzed Casein Medium

Titration in ml. $N/10$ NaOH.

Source of culture	Original culture medium	Base only	Base Biotin P.A.B.	Base B_1 P.A.B.	Base B_1 Biotin	Base B_1 Biotin P.A.B.	Base B_1 Biotin P.A.B. NA and PA
N.C.A. 3814	Hydrolyzed casein medium	1.55	1.80	2.00	3.15	7.95	8.70
N.C.A. 3814	Liver Infusion	1.80	1.90	3.25	4.00	6.60	7.00
Soybean Flour	Liver Infusion	1.25	1.65	2.30	2.50	6.40	7.45
Starch	Liver Infusion	1.50	1.45	2.90	2.90	5.70	6.10

from the strain cultured continuously in hydrolyzed casein medium. Since only two isolations of thermophilic anaerobes were tested it is possible that all strains of organism would not react in the same manner. These results indicate that under the conditions of this experiment continuous cultivation in hydrolyzed casein medium has not altered the metabolite requirements of the culture. Response to nicotinic acid and calcium pantothenate was not as marked in the N.C.A. culture carried in liver infusion and the culture isolated from starch as in the other two cultures. Though the response to the various metabolites was essentially the same, the N.C.A. strain cultured continuously in hydrolyzed casein media gave the greater acidity. Further subculturing of the strains in this experimental medium increased their ability to produce

acid. After five subcultures however they still failed to produce as much acid as the strain cultured continuously in hydrolyzed casein medium.

Summary

The nutritional requirements for maximum acid production by *Clostridium thermosaccharolyticum* N.C.A. strain No. 3814 have been determined in a hydrolyzed casein medium. Thiamine, biotin, and *p*-aminobenzoic acid were metabolites found to be essential. Nicotinic acid and calcium pantothenate gave a slight stimulative action. Under the conditions of this experiment, cocarboxylase could replace thiamine in the requirements of the organism. However, the thiazole fraction tested could only partially replace thiamine, and this organism did not respond to the pyrimidine fraction used.

A hydrolyzed casein medium in which the organism grows readily and produces a measurable acidity has been suggested.

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Riboflavin in the Nutrition of the Horse [1]

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Introduction

Investigations on the nutritional requirements of large animals such as the horse are attended with difficulties that are not encountered with small animals. This is probably one of the reasons why so little work has been done on the nutritional requirements of the horse. It is of interest and importance to have information on the nutritional requirements of animals of economic value. The fact that the B vitamins are produced by the symbiotic action of microorganisms in the rumen of ruminants is well established, but virtually nothing is known about the dietary requirements of the horse for these factors.

The physiological manifestations and behavior resulting from a riboflavin deficiency have been well established in various species of mammalia for which this vitamin is a dietary essential. The occurrence of one or more of these physiological responses in the horse restricted to a riboflavin-deficient diet, affords confirmatory evidence of the dietary essentiality of this vitamin for Equidae.

It is well recognized that a deficiency of riboflavin in the diet is reflected in a diminished urinary excretion of riboflavin. A marked decrease in the urinary excretion of riboflavin has been observed by various investigators (Vivanco, 1935; Fraser, *et al.*, 1940; Najjar and Holt, 1941; Axelrod, *et al.*, 1941) in experimental animals on restricted diets. Spies, *et al.* (1939) and Sebrell, *et al.* (1941) found that humans suffering from ariboflavinosis excreted much less riboflavin than normal individuals.

[1] The authors are indebted to Merck & Company for a generous supply of riboflavin and other synthetic vitamins, and to Anheuser-Busch Inc. for the brewers' yeast used in these investigations.

In our investigations with horses data have been secured on the influence of the riboflavin content of the diet, on the amount excreted in the urine, the amount in the blood, the growth, and general well being of the animals. Details of the growth performance of the animals as affected by the adequacy of the diet in roboflavin and certain other B vitamins will be reported in a separate communication.

Experimental

Shetland ponies were used in these investigations. With the exception of Guion who was about 22 months of age the others were about 9

TABLE I

*Experimental Rations**

Ration No.	IV	V	VI	VI-B
	per cent	*per cent*	*per cent*	*per cent*
Dried beet pulp	60	58	60	60
Corn, ground	29	25	29	29
Casein, purified	10	6	10	10
Brewers' yeast	—	10	—	—
Dicalcium phosphate	1	1	1	1
Riboflavin, synthetic mg./100 g. of ration	—	—	0.28	1.10
Total riboflavin content of ration, mg./100 g.	0.08	0.33	0.36	1.18

* Sodium chloride was fed *ad libitum*. A concentrate of vitamins A and D was fed at weekly intervals. Varying amounts of synthetic calcium pantothenate was included in the rations.

months of age at the beginning of the experiment. They were housed in individual stalls and a daily record kept of the feed consumption. At intervals of 2 or 3 times a week they were turned out in a paddock to exercise. A specially designed muzzle was used to prevent the horse from getting any feed while out of doors.

The composition of the rations used and their riboflavin content is shown in Table I. During the latter part of the experiment a new supply of beet pulp was secured which was considerably more bulky than the original lot. This necessitated reducing the proportion of beet pulp and adding a corresponding amount of corn to the ration. Rations IV and VI were changed to contain 40 per cent of beet pulp and 49 per cent of corn, while ration V was changed to contain 38 and 45 per cent of

beet pulp and corn respectively. This did not make any material change in the amounts of riboflavin as shown for the rations in the table. Rations V and VI were made up so as to contain approximately the same amounts of riboflavin.

The urine was collected quantitatively into a black bottle by means of a specially designed cage which excluded any contamination from feces. The plan was to collect the urine for 2 consecutive 24-hour periods. In a few instances where some of the urine was lost it was necessary to either keep the horse in the cage for a third day or use one 24-hour sample only. In this paper the values for the daily urinary excretion of riboflavin for horses represent the average for 2 days. The riboflavin was measured by the improved microbiological method described by Wegner, *et al.* (1942).

The horses fed ration IV did not consume as much feed as those that received added amounts of riboflavin and other of the B vitamins. Their daily feed consumption after 1 month on this ration rarely exceeded 1.8 kg. and averaged slightly less than 1.6 kg. per day. They weighed approximately 136 kg. with variations during the course of the experiment. On the basis of these figures the intake of riboflavin was slightly less than 10 μg. per kg. of body weight per day. This is considerably less than the requirements for mammalian species for which riboflavin is a dietary essential. Potter, *et al.* (1942) reported that the requirement of the dog for riboflavin is between 60 and 100 μg. per kg. of body weight per day. The requirements of the pig appear to be between 22 and 66 μg. per kg. of body weight per day (Hughes, 1940). The National Nutrition Committee recommends an allowance of 38.5 μg. of riboflavin per kg. of body weight per day for humans.

The effect of feeding a ration low in riboflavin on the amount excreted in the urine is shown in Table II. The initial value is the amount excreted before the horses were put on the experimental rations. At that time they were receiving feeds that would make up a typical horse ration, consisting of corn, hay, and a limited amount of grass. These animals were not changed directly from the stock ration to ration IV, but were first on one of the other experimental rations. The excretion of riboflavin on a stock ration is given in Table II as a guide to what horses of a comparable size might excrete on a typical ration. Comparison of the values on a stock diet with the amounts excreted when the animals were on rations V and VI (Table II) shows that they were of the same order. Furthermore, a change in the amount of riboflavin ingested is

reflected in the amount excreted in the urine within a period of a few weeks (Fig. 1).

It is not possible to measure by the microbiological method the amount of riboflavin in equine urine when the level is less than 2.5 μg. per 100 ml. Large amounts of urine must be added to each test tube to measure such small amounts of riboflavin, and excessive amounts of horse urine appear to be toxic to *Lactobacillus casei*. One month after the horses were placed on the ration containing not more than 80 μg. of riboflavin per 100 g. the daily urinary excretion of one of the horses was too low to measure, and for the other two it was less than one-fourteenth of the level when the animals were fed a stock ration. By the second month the urinary excretion of riboflavin was insufficient to measure in any of

TABLE II

Effect of Feeding Riboflavin-Low-Diet on Amount Excreted in the Urine

Time	Cleburne	Guion	Rondo
months	*mg./24 hrs.*	*mg./24 hrs.*	*mg./24 hrs.*
Initial value			
Stock diet	1.05	2.14	1.76
1	<0.03	0.15	0.08
2	<0.03	<0.03	<0.03
3	<0.03	<0.03	<0.03
4	<0.03	<0.03	0.08
5	<0.03	<0.03	0.05
6	<0.03	<0.03	<0.03

the animals. The excretion of riboflavin continued at these relatively low levels until the animals were taken off the ration at the end of 6 months.

It might be pointed out that the urinary excretion of riboflavin of the horses on ration IV was only a fraction of the intake. This is different than with nicotinic acid where the urinary excretion may exceed the intake (author's unpublished data). As has previously been mentioned the urinary excretion of riboflavin by animals for which it is a dietary essential is closely correlated with the intake, and clinically, low urinary values are characteristic of ariboflavinosis. On the basis of this as a criterion the evidence strongly indicates that riboflavin is a dietary essential for the horse just as it is for all other monogastric mammals that have been studied. The fact that a source of some of

the B vitamins including riboflavin was necessary in the ration for satisfactory growth performance of the horses is further confirmatory evidence of its dietary essentiality for Equidae.

Quantitative requirements. Efforts to measure the quantitative requirements of the horse for riboflavin were made using the amount excreted in the urine and the growth performance as criteria. While these data are not conclusive the evidence indicates that the requirements of the horse for riboflavin is of the same order as for humans. Sebrell, *et al.* (1941) studied the urinary excretion of riboflavin in humans on controlled diets and concluded that the requirement of man is between 35 and 60 μg. per kg. of body weight per day.

TABLE III

Urinary Excretion of Riboflavin of Horses Fed Yeast and Synthetic Riboflavin

Months on ration	Ration V Containing Yeast 0.33 mg. Riboflavin/100 g.		Ration VI Synthetic Riboflavin 0.36 mg. Riboflavin/100 g.	
	Cleburne	Rondo	Bastrop	Dallas
	mg./24 hrs.	*mg./24 hrs.*	*mg./24 hrs.*	*mg./24 hrs.*
1	1.30	1.34	2.00	1.87
2	1.21	1.50	1.47	0.95
3	1.03	2.03	1.86	1.39
4	1.13	2.07	0.70	1.73
5	2.88	2.77	1.66	1.28
6			1.20	0.60
7			1.80	1.05
8			1.33	1.57

In Table III is shown the daily riboflavin excretion of horses fed rations containing 0.33 and 0.36 mg. of riboflavin per 100 g. of ration. The essential difference between these two groups is that the riboflavin in ration V was from natural sources, while No. VI is the basal ration supplemented with synthetic riboflavin. The daily intake of riboflavin was approximately 44 and 48 μg. per kg. of body weight on rations V and VI respectively. Over a period of 5 months during which ration V was fed and 8 months on ration VI there was some individual variation in the excretion of riboflavin. However, they were fairly consistent. The variations were no greater than Sebrell, *et al.* (1941) observed with humans on controlled diets. They found that the daily urinary excretion of riboflavin of 7 subjects ranged from 130 to 810 μg. with an average

of 357 μg. daily. When they fed 60 μg. of riboflavin per kg. of body weight per day the excretion increased to over 1000 μg.

An examination of Tables II and IV shows that when the horses received rations V and VI providing an intake of 43.7 and 47.6 μg. per kg. of body weight daily respectively that the urinary excretion was of the same order as when they were on the uncontrolled stock diet. Increasing the intake of riboflavin to 156 μg. per kg. of body weight daily resulted in a marked increase in the amount excreted in the urine. The horses receiving rations V and VI made satisfactory gains in weight. While our data are not adequate to give an exact figure for the riboflavin requirements of the horse, it is apparent that 44 μg. per kg. of body weight per day is adequate. The evidence indicates that the

TABLE IV

Summary of Relation of Intake to Outgo of Riboflavin

Ration	Per 100 g. ration	Daily Intake		Urinary Excretion	$\frac{\text{Intake}}{\text{Outgo}}$ =
		Total	Per kg. body weight		
	mg.	*mg.*	*μg.*	*mg./24 hrs.*	
IV	0.08	1.28	9.4	<0.03	42.7>
V	0.33	5.94	43.7	1.73	3.44
VI	0.36	6.48	47.6	1.40	4.61
VI-B	1.18	21.24	156.2	8.69	2.44

riboflavin requirements of the horse are somewhat less than for the dog and probably of about the same order as for humans.

The ratio of the riboflavin intake to the amount excreted in the urine decreases as the amount ingested increases. On the basal ration the ratio of riboflavin intake/outgo exceeds 1 to 43, while on an adequate intake of riboflavin the ratio is in the order of 1 to 4. On low levels of intake a larger proportion of the riboflavin appears to be retained by the body, probably for normal physiological functions.

That an increase or decrease in the riboflavin intake is promptly reflected in the amount excreted in the urine is shown in Fig. 1.

Within a period of one month after changing the amount of riboflavin in the ration, the amount in the urine appears to approach a relatively constant value for that particular level of intake. In humans Sebrell, *et al.* (1941) found that the amount of riboflavin excreted in the urine

reached a fairly constant value within 20 days after changing the amount ingested.

Riboflavin content of blood. Each time the urine was collected blood was drawn from the jugular vein and assayed for riboflavin. Since the riboflavin content of the blood failed to show a consistent relationship to the intake, the detailed data are not reported here. There was no evidence to indicate that the amount of riboflavin in the blood can be used as a criterion as to the nutritional state of the animal or the adequacy of the diet in riboflavin. These observations are in accord with those of Axelrod, Spies, and Elvehjem (1941) on humans. They found

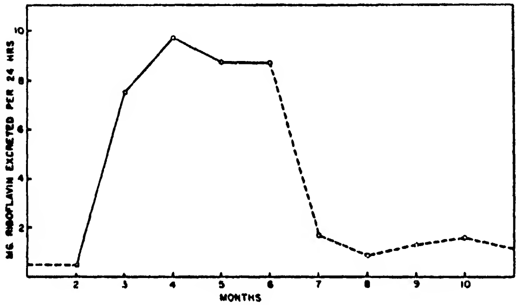

Fig. 1

Effect of the Ingestion of Different Levels of Riboflavin on the Amount Excreted in the Urine

Broken line represents riboflavin intake of 43.7 μg. per kg. of body weight per day and solid line 156.2 μg. per kg. of body weight per day.

that the blood riboflavin values of patients with varying degrees of riboflavin deficiency were no lower than the values of normal subjects.

Whole horse blood contained an average of 25 μg. of riboflavin per 100 ml. Over a period of several months there was considerable variation in the values for individual animals even when the dietary regimen was constant.

Summary

Horses were fed experimental rations furnishing varying amounts of riboflavin. On the basis of the amounts of riboflavin excreted in the urine, evidence is presented that this vitamin is a dietary essential for the horse. A daily intake of 44 μg. of riboflavin per kg. of body weight per day adequately satisfied the requirements of horses under the experimental conditions described.

The amount of riboflavin excreted in the urine is closely correlated with the intake. The amount of riboflavin excreted daily by horses fed a ration furnishing 9.4 μg. of riboflavin per kg. of body weight is usually less than 0.03 mg. per day. With an adequate intake the daily urinary excretion of the horses used in these experiments was in the order of 1.5 mg. per day.

The riboflavin content of the blood of horses subjected to the different dietary regimens failed to show any consistent relationship to the intake.

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Rapid and Accurate Calibration of Warburg Manometers

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For the calibration of Warburg manometers it is necessary to determine the volume of the gas space in the vessel, including that of the connecting- and manometer-tubes down to the half way mark (150 or 180 mm.). Most laboratories determine this volume following the procedure described by Dixon (1), which is time consuming as it requires two careful adjustments of the mercury volumes by adding or removing small amounts.

With the procedure described here, it is possible to calibrate a Warburg manometer in approximately 5 to 10 minutes.

Step 1: Fill the side vessel with mercury and attach it to the manometer. The amount of mercury used can vary within reasonable limits, but it should be enough to fill the side arm completely and reach or approach the manometer capillary at 1 in Fig. A.

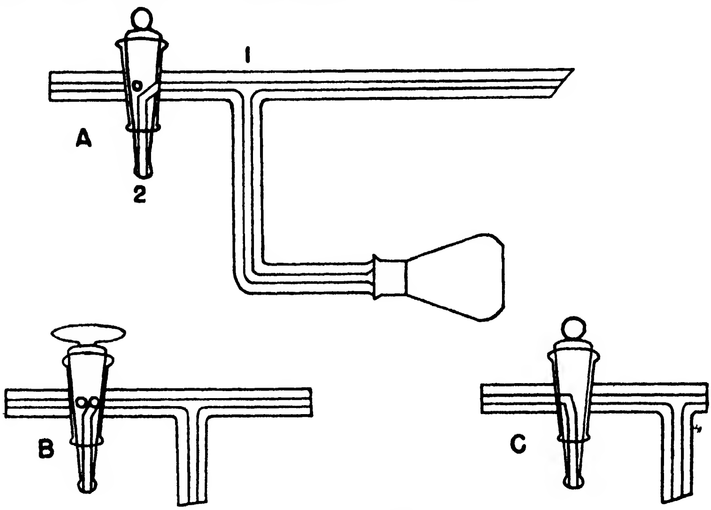

Fig. A B C

Step 2: Rest the manometer (with Hg-filled side vessel) in a horizontal position, for example on a tripod, and attach a mercury reservoir at 2. Such a reservoir is simply a 10–15 cm. piece of vacuum tubing, one end of which is closed by a screw clamp. Attach a second screw clamp to the Hg-filled rubber tubing and with the manometer stopcock in the position as shown in Fig. A, gradually tighten this screw clamp. The mercury column should slowly move through the stopcock bore, join the column from the side arm and should be stopped exactly at the desired half way mark (150 or 180 mm.).

Step 3: Turn the manometer stopcock 1/8 turn (45°) so that both bores face the operator (Fig. B). Detach the mercury reservoir. Lift the instrument on the left side to an angle of about 45° with the table surface. The mercury column will gradually move away from the stopcock towards the bend of the U-tube. When it has moved about 2–3 cm., complete the stopcock turn to 180°, so that the Hg which remained in the stopcock bore drops out (Fig. C). Return the manometer to the previous horizontal position.

Step 4: Put a weighed beaker under the tail of the stopcock (2 in Fig. A) and turn the stopcock back 180° to the position shown in Fig. A. This will bring the mercury from the graduated capillary into the beaker. Detach the side vessel (over a porcelain dish) and add the mercury to that in the beaker. Note the temperature of the mercury and weigh.

Remarks: The stopcock and the ground joint holding the side vessel are not to be lubricated for the calibration. The whole procedure is described here at length to avoid misunderstanding; actually it is very simple.

Accuracy: In duplicate and triplicate determinations the weight of the mercury agreed to within 100 mg. or less, corresponding to volumes of less than 0.01 ml.

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Letter to the Editors

Anthranilic Acid and the Biosynthesis of Indole and Tryptophan by Neurospora*

Indole (1, 2) and anthranilic acid (2) have been proposed as intermediates in the biosynthesis of tryptophan by certain bacteria. Since all bacteria which were tested on both compounds and which grew on either were able to utilize both, the order of these compounds in the biosynthesis of tryptophan could not be established (2). The direct biosynthesis of tryptophan from indole and serine (3) makes it more probable that anthranilic acid is the precursor of indole.

More direct evidence is now available from results obtained with specific biochemical mutant strains of *Neurospora crassa*, the general origin of which is described elsewhere (4). Strain 10575, a tryptophan-deficient mutant which grows well on indole, is unable to use anthranilic acid. However, a second genetically different strain can use either indole or anthranilic acid. The chain of reactions leading to the synthesis of tryptophan is evidently broken in one case before anthranilic acid, and in strain 10575 between anthranilic acid and indole. It appears therefore that anthranilic acid is an intermediate in the biosynthesis of indole and tryptophan in *Neurospora*.

This hypothesis is proved by the demonstration that strain 10575 produces a relatively large amount of anthranilic acid, which accumulates in the medium. This production and accumulation of anthranilic acid is the unique property of this mutant strain, and is an example of the accumulation of an intermediate compound the utilization of which is genetically blocked. The material isolated from the culture medium of strain 10575 melted at 141–142°C. (uncor.) and gave no depression when mixed with an authentic sample of anthranilic acid.

A few compounds which might be intermediates in the synthesis of anthranilic acid have been tested on the anthranilicless mutant strain. Pimelic acid, salicylic acid, benzoic acid, and aniline were inactive in promoting growth.

In an attempt to determine the course of synthesis of indole from anthranilic acid, a number of other compounds have been tested. *o*-Ami-

* Work supported by grants from the Rockefeller Foundation.

nophenylacetaldehyde has been suggested as a precursor of indole in the bacterial breakdown of tryptophan (5). Since it forms indole spontaneously, a direct test as an intermediate would be meaningless. However, the low activity of *o*-aminophenylethanol for both mutant strains makes this an improbable intermediate in Neurospora, and *o*-aminophenylacetic acid is inactive. *o*-Carboxyphenylglycine has approximately the molar activity of anthranilic acid. It is active only for the anthranilicless mutant while phenylglycine is inactive for both mutants. The synthesis of indole by strain 10575 appears to be blocked in the reaction immediately following anthranilic acid. If this is true, the anthranilic acid derivative, since it is inactive for mutant 10575, cannot be an intermediate in the synthesis of indole from anthranilic acid. *o*-Carboxyphenylglycine would then owe its activity for the anthranilicless mutant to a conversion to anthranilic acid.

To establish a compound as an intermediate in the synthesis of indole from anthranilic acid would require evidence analogous to that presented here which proves that indole is an intermediate in the synthesis of tryptophan from anthranilic acid.

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December, 1943.

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BOOK REVIEWS

Chemistry and Physiology of Vitamins. By H. R. ROSENBERG, Sc.D., E. I. DuPont de Nemours & Company. Interscience Publishers, Inc., New York, N. Y., 1942. xix + 674 pp. Price $12.00.

This monograph is an up-to-date, comprehensive treatment of the important developments in vitamin research. The opening chapter defines vitamins and distinguishes them from the "vitagens", a name proposed for such compounds as the essential fatty and amino acids, choline, etc. This is followed by a general discussion of the schematic outline used in developing the later chapters on the individual vitamins.

Fourteen vitamins or vitamin groups are rigidly surveyed. Where information is available, each is discussed with reference to chronology, occurrence, isolation, properties, constitution, synthesis, industrial methods of preparation, biogenesis, specificity, determination, standards, physiology, and pathology. One chapter is devoted to non-identified vitamins and another to the vitagens. A novel feature is the patent index, which lists practically all the patents pertaining to vitamins.

The reader will look in vain for tabulations of the vitamin content of foods. This omission is defended on the basis of the inadequacies of available data and the desirability of teaching the public in general terms the merits of well-balanced diets. The subject of vitamin requirements is rather sketchily and non-critically presented, and in this respect will disappoint those seeking comprehensive information. Nor is the discussion of methodology adequate for those whose interests lie primarily with problems of assay; many of the listed procedures have serious practical limitations. In a few instances the reader may question the validity of some of the conclusions reached by the author. Thus, the statement that "there is ample evidence that Vitamin D_3 is somewhat more effective in man than Vitamin D_2" will not receive universal acceptance at the present time.

As indicated by its title, the volume has greatest merit in the chemical and physiological features. The close association of the author with the activities of Ruzička and Reichstein is reflected in the excellence of the purely chemical aspects. Detailed discussions profusely illustrated with formulas and equations adequately describe the achievements made in the fields of constitution and synthesis. The completeness of this treatment somewhat shades that devoted to physiology, a defect which is somewhat modified by the extensive bibliography.

The work is well organized and presented, and will serve as an important adjunct to many fields of biochemical and physiological research.

JOHN S. ANDREWS, Minneapolis, Minn.

Physical Chemistry for Students of Biochemistry and Medicine. By EDWARD S. WEST, Professor of Biochemistry in the University of Oregon Medical School. The Macmillan Co., New York, N. Y., 1942. xiv + 368 pp. Price $5.75.

The intention stated in the preface is to provide a relatively non-mathematical treatment of physical chemistry for students with a minimum of chemistry and mathematics. It will, therefore, be most useful for premedical or medical students, or for students of general biology. Students of biochemistry might reasonably be supposed to be prepared for a more rigorous and mathematical treatment.

In the early chapters of the book, such subjects as atomic structure and valence are considered. In this connection, the electron structure of the atom and its relation to valence are considered, together with a brief discussion of nuclear structure and isotopes. This is followed by a discussion of gases and their properties. From the solubility of gases, the general concepts of solubility are introduced.

The fourth chapter is devoted to osmotic pressure. The analogy with the gas law is pointed out, but in a non-mathematical discussion the relation between vapor pressure and osmotic pressure is also considered. In this connection, freezing point depression, boiling point elevation, and distribution between phases are also discussed.

In the chapter on electrolytic dissociation and the mass law the conductance of solutions is taken up in a very general way, as an adjunct to the development of the ideas of complete and incomplete dissociation and of activity. The idea of reversible reactions is introduced, and the equilibrium constant is derived from kinetic considerations.

From here, West proceeds logically to acids, bases and buffers. The Brönsted conception of acids and bases is used, which greatly facilitates the discussion. The classical conception of basic dissociation is also used in certain connections, which is probably justifiable in that students are accustomed by most elementary courses to think, in that way. The ideas of pH and buffers are thoroughly developed, with considerable space devoted to the mechanics of the calculations involved.

Chapter VII is concerned with the determination of pH, and the limitations of the various methods. The use of indicators in buffer solutions or as bicolor standards (but not with the colorimeter), the hydrogen electrode, the quinhydrone electrode, and the glass electrode are discussed.

In the chapter on the colloidal state and membrane phenomena, the types and properties of colloidal systems are considered. The principles of ultrafiltration, diffusion and Brownian movement, and the ultracentrifuge are briefly treated in connection with the determination of the size of colloidal particles. The nature of the Helmholtz-Gouy double layer and its relation to streaming potential, electro-endosmosis, and the stability of colloids is discussed. The properties of surfaces, including surface tension, adsorption, and the nature of surface films are considered at some length. Viscosity is discussed briefly, and membrane phenomena, particularly the Donnan equilibrium, in more detail.

Oxidations and reductions are approached from the standpoint of electron transfers. The use of electrode reactions in oxidation reduction studies is amply discussed and illustrated, both with non-biological and biological examples.

The book is concluded with a brief introduction to reaction velocity. The expressions for reaction rate are developed using simple collision theory. The function of catalysts is given brief consideration.

It seems that the book accomplishes its purpose quite satisfactorily. The

discussions are clear, and developed in such a way that the student should be able to follow them readily. Objections may be raised to certain points, such as the use of fog for an example of a solution of a liquid in a gas (pp. 87), or the statement that the concentration of a solid may be taken as constant because the amount is great relative to the amount in solution (pp. 125). The inclusion of "pure gastric juice" in Table 11 can hardly be justified. It also seems confusing to the reviewer to call liquid-in-liquid colloidal systems emulsoid or lyophilic colloids,—particularly when emulsions are discussed under a separate heading. In the section on diffusion, it is not noted that the relations $D = \frac{kT}{6\pi\eta r}$ and $D\sqrt{M} = K$ are contradictory. It would probably have been better not to include the latter expression, except as it applies to gases. In the discussion of adsorption, the statement is made that adsorption is fastest at low temperatures. It would seem that this statement needs some qualification or a more expanded discussion.

The reviewer is also inclined to feel that some space should have been devoted to the conventions of sign for electrode reactions. Possibly this would only confuse students who are not expected to make much practical use of electrode reactions, but on the whole, more serious confusion might be avoided. The electrode potentials are given according to the rather common convention that they represent the sign of the electrode of the right half of a cell against a standard hydrogen electrode, so that the sign is positive if the electrode is positive to the hydrogen electrode. However, the electrode reactions corresponding to these potentials are written

$$x \rightarrow x^{+} + e$$

This could lead to some confusion if a student were using the conventional table of the electromotive series and corresponding potentials as it is commonly given in the chemical literature. It would seem preferable if the electrode reactions were written

$$x^{+} + e \rightarrow x$$

to correspond with the sign of the potential used.

It is relatively easy to find fault with any book, or to suggest changes that appeal to a particular reviewer. For example, the rather common use of electrophoretic methods might justify some discussion in a book of this sort. Further considerations might have been introduced in the discussion of viscosity, such as the influence of shape on the viscosity of solutions of a particular molecule. In view of the use to which colorimetry is put in medicine and biology, some discussion of this subject might also have been included. However, such additions could not well be made without restricting some other portions or without increasing the price of the book. While the reviewer welcomes a supplementary text of this nature for medical biochemistry, the price of this book seems rather high for such a purpose. Professor West's book will probably find most use where a short course in physical chemistry can be given to medical students, or in premedical courses in physical chemistry.

John W. Mehl, Los Angeles, Cal.

Introduction to Organic and Biological Chemistry. By L. Earle Arnow, Ph.D., M.D., Director of Biochemical Research, Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa., and Henry C. Reitz, Ph.D., Assistant Chemist in the Western Regional Research Laboratory, United States Department of Agriculture, Albany, California. The C. V. Mosby Company, St. Louis, Mo., 1943. 736 pages. Illustrated. Price $4.25.

Perhaps the most controversial part of this entire volume is the opening paragraph of the preface. The authors assume that it is desirable, or at least frequently necessary, to teach organic and biological chemistry as one brief course. The reviewer doubts the desirability and deplores the necessity of such a practice. He is willing to admit, however, that if such a course must be taught it is probably helpful to have a text designed for the purpose. For the rest of this review it will be taken for granted that such a course must be given.

Obviously, the prospective authors of a book with the indicated title are confronted with a vast array of material. If even the bare outlines are to be presented in a manner that will hold a student's interest and enable him to grasp the essential facts, considerable skill is required. The authors have performed this difficult feat well. The book is profusely illustrated, and the text contains much explanatory material pointing out the application of the facts being considered to industrial procedures, public health, the practice of medicine, etc. Even advice about how to master the facts is included and a group of study questions is provided at the end of each chapter. References to the literature are included at the end of the chapters, but no attempt is made to cite or evaluate the evidence for the statements presented in the text.

The book is divided into three sections. Section I contains only two brief chapters on chemical fundamentals. The next nineteen chapters deal with organic chemistry and the closing nine chapters with biochemistry Sections of the Heinz nutrition charts are reproduced in the appendix. The proportion of nineteen to nine does not give a true indication of the amount of attention devoted to biochemistry. Some of the chapters, such as the ones on lipids and polysaccharides that are included in the organic section might equally well be placed in the biochemistry section. The saving of space obtained by having some chapters serve both sections instead of repeating them, as one would have to do in separate books on organic and biological chemistry, is one of the important justifications of the present arrangement.

The emphasis on organic chemistry has the disadvantage of causing, or permitting, the neglect of elementary physical chemistry. Such neglect shows itself in a number of ways. Thus, one might expect a better definition of a base than that it forms hydroxyl ions in solution. The term, buffer, is not mentioned and the whole process of pH regulation in the tissues is treated very sketchily or ignored. Even such concepts as dissociation constants are omitted, except by implication, and as a result such chapters as the one on proteins and amino acids suffer considerably.

Probably each reader will wish that this or that subject had been treated in more detail, but for the over-all effect the allotment of attention is probably just. The book is well printed and substantially free of errors. With the limitations noted, the work can be recommended.

C. V. Smythe, Philadelphia, Pa.

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A Simplified Fluorometric Method for Riboflavin in Meat[1]

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Received October 11, 1943

Introduction

Existing methods for the determination of riboflavin are in general limited in value because, when applied to food products other than those used in developing the method, they may not be specific for riboflavin or else all of the riboflavin present in the food may not be estimated. It was not known how satisfactory they would be when applied to meat. This paper reports the modifications which proved satisfactory for meat.

A fluorometric method seemed less laborious for routine analyses and was selected for trial by the authors. The possible sources of error which were considered were: incomplete extraction of the riboflavin, interference caused by the presence in the purified extract of varying amounts of colored or fluorescent impurities, and losses occurring during the various processes of purification.

Extraction of riboflavin has been accomplished in different ways: refluxing in 0.25 N H_2SO_4 (1), heating on the steam bath in dilute H_3PO_4 (2) or 0.04 N H_2SO_4 (3), autoclaving in 0.1 N HCl (4) or in distilled water followed by digestion with takadiastase (5), but Cheldelin (6) reported that with animal tissues such as hog heart and beef brain proteolytic enzymes gave more complete extraction than did autolysis or autoclaving with either water or dilute acids.

Removal of interfering substances has been accomplished by various procedures, some of them very laborious. Ferrebee (7) used an ad-

[1] This work was supported in part by a grant from the National Live Stock and Meat Board made through the National Research Council.

[2] Cooperative project between the Division of Rural Home Research and the Nutrition Laboratory, Department of Animal Husbandry.

sorption column, recommending a heat-activated floridin and a synthetic material, supersorb, as superior to crude floridin earth for adsorbing riboflavin. The riboflavin was eluted from the adsorbing column with an aqueous solution of pyridine and acetic acid. The resulting eluate, however, was not free from interfering substances and was treated with $KMnO_4$ and H_2O_2. Conner and Straub (3) selected supersorb to adsorb the riboflavin in their method for determining riboflavin and thiamine on the same sample. Hodson and Norris (1) omitted adsorption but used a preliminary reduction with $Na_2S_2O_4$ and $SnCl_2$, followed by mild oxidation with air. They reported that, in the presence of $SnCl_2$, none of the interfering substances encountered up to that time had been readily reoxidized to the fluorescent form, whereas riboflavin was. Swaminathan (8), however, was unsuccessful in repeated attempts to use this technique. He found that the interfering blue fluorescent materials behaved in the same manner as riboflavin when acted upon by $Na_2S_2O_4$ and $SnCl_2$.

Traces of stable interfering pigments which are difficult to remove may alter the fluorometric response of the riboflavin in the sample. Hodson and Norris (1) and Andrews, *et al.* (5) overcame this difficulty by adding a known amount of standard riboflavin to the unknown. With this procedure the interfering substances should affect the standard in the same way as the riboflavin originally present in the unknown.

The blank was obtained by Chapman and McFarlane (9) by reduction of the riboflavin with a benzyl alcohol solution of $SnCl_2$ carried out in a separate cuvette. Hodson and Norris (1) added a solution of $Na_2S_2O_4$ to the cuvette containing the unknown and used a correction factor to compensate for change in volume caused by dilution. Andrews, *et al.* (5) used 10–20 mg. of solid $Na_2S_2O_4$ with no correction factor. Since the fluorescence of riboflavin is destroyed at pH 11 and only one drop of saturated NaOH is required to bring the solution in the cuvette to approximately pH 11, this seemed to the authors to be a convenient way of obtaining a blank.

Method

Extraction of riboflavin from meat is accomplished with a combination of enzymes, papain and takadiastase[3] at pH 4. Impurities in the

[3] The same method of extraction is used in this laboratory for thiamine, riboflavin, nicotinic acid, and pantothenic acid so that these vitamins may be determined on aliquots of the same extract.

resulting extract appear to be mainly fat and protein. Fat is removed effectively with chloroform. Most of the remaining protein material is removed by precipitation at pH 6.8–7.0.

Fluorometric measurements are made on the unknown at pH 6.8–7.0, at which riboflavin exhibits maximum fluorescence. The necessity of removing traces of interfering pigments is overcome by adding a known amount of the standard riboflavin to the unknown. A blank then is obtained by adding one drop of a saturated solution of NaOH to bring the solution to pH 11 at which the fluorescence of riboflavin is destroyed.

It is necessary to carry out the entire analysis under subdued light.

Preparation of Extract. A 4 g. sample of finely ground meat is weighed into a 250 ml. flask.[4] To the flask is added 100 ml. of 0.1 *M* sodium acetate buffer (pH 4), 0.1 g. each papain (Difco) and taka-diastase, and 4 drops concentrated HCl. The contents of the flask are stirred at 300 r.p.m. for 2 hours. A few drops of benzene are added and the flasks placed in an oven (45–50°C.) for approximately 15 hours.

Removal of Interfering Substances. Three ml. of chloroform are added, the flasks shaken vigorously for 2 minutes and then allowed to stand until the chloroform settles to the bottom. The digest is decanted and filtered first through filter paper[5] and then through a Hirsch sintered glass funnel covered with a very thin layer of Filter-Cel. An aliquot of 50 ml. is adjusted to pH 6.8–7.0 with NaOH, and allowed to stand overnight in the refrigerator. It is filtered through filter paper[5] and the precipitate washed several times with distilled water. The volume of filtrate and washings is made up to 150 ml.

Fluorometric Readings. The photofluorometer used in this laboratory is a Coleman Model 12, fitted with filters B-2 and PC-2. A compensating transformer is used to deliver constant voltage to the mercury lamp. The instrument is standardized with a fluorescein solution (0.1 μg./ml. of water) to give a deflection of 30–40 scale divisions.

A 15 ml. aliquot of extract is pipetted into a cuvette and the fluorometric reading (A) taken. One ml. of standard riboflavin solution containing 1 μg./ml. is added. After agitation, the reading (B) is taken. One drop of saturated NaOH is added which brings the solution to approximately pH 11 and destroys the riboflavin. After agitation, the

[4] Centrifuge bottles of 250 ml. capacity were used.

[5] Whatman No. 4 filter paper was used.

reading of the blank (C) is taken. The riboflavin content of the sample is calculated from the equation:

$$\left(\frac{A - 1.06\ C}{B - 0.94\ A} \times 0.063 \times \frac{300}{\text{Wt. of sample}}\right) - \begin{matrix}\text{Enzyme}\\ \text{blank}\end{matrix} = \mu g./g.$$

where A, B, and C are the instrument readings in scale divisions, the numerical values 1.06 and 0.94 are correction factors which compensate

TABLE I

Recovery of Added Riboflavin

Sample number	Riboflavin content	Riboflavin added	Riboflavin found	Riboflavin recovered
	µg./g.	*µg./g.*	*µg./g.*	*per cent*
1	2.00	5.0	6.80	96
2	2.12	3.4	5.42	97
3	2.15	3.4	5.44	97

TABLE II

Comparative Results by Fluorometric and Microbiological Procedures for Raw Beef

Sample number	Fluorometric	Microbiological
	µg./g.	*µg./g.*
1050	1.37	1.45
1051	1.32	1.51
1052	1.26	1.47
1053	1.04	1.24
1054	1.16	1.29
1055	1.25	1.55
1056	1.05	1.47
1057	1.12	1.45
1058	1.29	1.43
Mean	1.21	1.43

for dilution with the added solution of pure riboflavin, 0.063 is a contraction of

$$\left(\frac{\text{Vol. at A}}{\text{Vol. at B}} \times \frac{\mu g.\ \text{riboflavin in standard}}{\text{Vol. of aliquot}}\right),$$

and 300 is a contraction for the original volume of the extract (100) times the dilution factor $\left(\frac{150}{50}\right)$.

Recovery Tests

One criterion of the adequacy of a method is the recovery of known amounts of riboflavin added before extraction. Recoveries from raw beef of 96–97% (Table I) indicate that only negligible losses of crystalline riboflavin occur during this procedure.

Comparison with Microbiological Procedure

In order to test the validity of the simplified fluorometric method described here, parallel assays were made on the same extract from 9 roasts of beef using for the comparison the microbiological method of Snell and Strong (10) as modified by Wegner, *et al.* (4).

Riboflavin values obtained by the two methods are given in Table II. Although the results by the simplified fluorometric method were slightly lower than by the microbiological the authors consider that good agreement between the two methods was obtained.

Summary

A simplified fluorometric method for determining riboflavin in meat is presented. Interfering substances are removed by extraction with chloroform and precipitation at the appropriate pH. Interference from traces of remaining pigments is avoided by adding a known amount of riboflavin directly to the cuvette containing the unknown. A blank is provided by adjusting the solution in the cuvette to pH 11.0 which destroys the fluorescence of riboflavin.

The recovery of riboflavin added prior to digestion was 96–97%. The simplified fluorometric method gave results for beef which were in good agreement with those by the microbiological method.

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The Water-Soluble Polysaccharides of Sweet Corn

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Introduction

Morris and Morris (1) isolated from *Zea Mais* (variety Golden Bantam) a polysaccharide which they concluded was indistinguishable from glycogen. They based their evidence, among other things, upon the similarity of the cupric chloride crystallization patterns formed in the presence of the new polysaccharide and glycogen, respectively. The new polysaccharide gave a reddish color with iodine and was digested by malt amylase at nearly the same rate as glycogen from different sources. This rate was slower than that for starch and erythrodextrin prepared from corn starch. Hassid and McCready (2) studied the polysaccharide of Morris and Morris and concluded that it was similar to glycogen, since after methylation followed by hydrolysis, it yielded a proportion of tetramethylglucose that corresponded to a repeating unit of 12 glucose molecules. They found the repeating length in corn starch to be 25 glucose molecules.

We have repeated the isolation of the glycogen-like polysaccharide, using the technique of Morris and Morris as well as a method of our own. At first we believed that soaking grains of corn in water, as practiced by Morris and Morris (1) might lead to alterations of the polysaccharides, but we have found that such alterations do not occur. We have discovered that Golden Bantam, Carmel Cross, and Marcross varieties of sweet corn contain two water-soluble polysaccharides which are quite distinct from corn starch. One of these which we shall call polysaccharide no. 2, corresponds to the glycogen of Morris and Morris. It is not precipitated by adding 2 volumes of glacial acetic acid to its dilute solution, as they found. We have noted that its solutions are about as opalescent as are solutions of animal glycogen. It gives a somewhat more intense brown color with iodine than does glycogen prepared from

beef liver by the method of Sahyun and Alsberg (3). If one volume of ammonium sulfate is added to one volume of the polysaccharide solution the color produced upon adding iodine is very intense. Glycogen solutions likewise give an intense color with iodine if an equal volume of saturated ammonium sulfate is added. Our most dependable method for differentiating between polysaccharide no. 2 and animal glycogen is as follows:

To 10 cc. of a one-half per cent solution of the material add 10 cc. of saturated ammonium sulfate. Mix and filter. Add to the filtrate one drop of 0.01 N iodine solution in potassium iodide and mix. If the material is animal glycogen a yellow color will be produced. If the material is polysaccharide no. 2 a pink color will appear.

The second water-soluble polysaccharide in sweet corn we shall call polysaccharide no. 1. This fraction, which Hassid and McCready apparently considered to be starch, is quite distinct from polysaccharide no. 2, as well as from starch amylose and starch amylopectin. It gives a blue color with iodine, but the blue is not nearly so intense as that given under comparable conditions by corn starch amylose. It is readily soluble in cold water, giving solutions which are extremely opalescent. It is readily precipitated by adding 2 volumes of glacial acetic acid to its aqueous solution and after two such precipitations no more polysaccharide no. 2 can be removed. Polysaccharide no. 1 is entirely converted to *d*-glucose upon hydrolysis with 5 per cent sulfuric acid, as indicated by the finding that the specific rotation of the product is +52.5°. We have found that air-dried Golden Bantam corn contains about 30 per cent of combined polysaccharides no. 1 and no. 2. Of this about two-thirds is no. 2. The quantity of true starch found was 21 to 25 per cent. We suggest that the polysaccharide of Morris and Morris, polysaccharide no. 2, be called "phytoglycogen" and that our polysaccharide no. 1 be called "glycoamylose."

Experimental

Polysaccharides no. 1 and 2 were usually extracted by stirring 200 g. of finely ground Golden Bantam, Carmel Cross, or Marcross sweet corn with 600 cc. of cold 10 per cent trichloroacetic acid. The liquid was pressed out from cheesecloth and centrifuged at once. The supernatant was filtered through cotton, and to each volume 2 volumes of 95 per cent alcohol were added. The precipitate, which contained both polysaccharides, was centrifuged off, washed repeatedly with 95 per cent alcohol and dried at 40°C. To separate polysaccharides 1 and 2 some of the material was dissolved in water and mixed with 2 volumes of glacial acetic acid

according to the procedure of Morris and Morris (1). The precipitated polysaccharide no. 1 was centrifuged off, immediately washed with alcohol until free from acid and dried at 40°C. Polysaccharide no. 2 was precipitated from the supernatant solution by adding 1.5 volumes of 95 per cent alcohol and centrifuging. This precipitated material was washed with alcohol to free it from acid and was then dried at 40°C.

For the purpose of determining the percentages in sweet corn of polysaccharides no. 1 and 2 and starch we ground weighed amounts of whole corn grains, either air-dried or water-soaked, in 10 per cent trichloroacetic acid by means of a "Powermaster" blender. Each sample was ground for only a few minutes at a time in order to avoid heating. The ground material was centrifuged and the precipitate was ground and extracted again and again until only negligible quantities of polysaccharides were extracted, as shown by the iodine test. Polysaccharides no. 1 and 2 were then precipitated from the combined extracts by adding 2 volumes of 95 per cent ethyl alcohol. The precipitate was washed with alcohol and dried at room temperature *in vacuo* over sulfuric acid. Polysaccharide no. 1 was separated from polysaccharide no. 2 by precipitating twice with acetic acid, according to the method of Morris and Morris (1). The fractionation was completed as rapidly as possible, and the fractions were washed free from acid by alcohol and then dried *in vacuo*. We have found that if commercial corn starch is ground in a blender with 10 per cent trichloroacetic acid only a negligible amount of the initial dry weight is extracted.

Starch was isolated from the centrifuged residue by making repeated extractions with boiling water. The aqueous extracts were filtered by suction through filter paper, precipitated by 2 volumes of alcohol, washed with alcohol and dried *in vacuo*.

Summary

A test is described whereby animal glycogen can be distinguished from the polysaccharide isolated from sweet corn by Morris and Morris. A new polysaccharide has been shown to be present in three varieties of sweet corn. This substance gives a slight blue color with iodine. It is soluble in cold water and its solutions are intensely opalescent. Although an anhydride of *d*-glucose, it is distinct from starch amylose, amylopectin, glycogen, and the polysaccharide of Morris and Morris.

We wish to express our gratitude to the Rockefeller Foundation for the financial assistance which made this research possible.

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The Preparation of Glucose-1-Phosphate

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Received November 22, 1943

Introduction

Hanes (1) prepared glucose-1-phosphate in quantities as great as 80 g. at a time by allowing phosphorylase from plants to act upon soluble starch and phosphate. At first he employed phosphorylase prepared from Laxton's Progress peas. Later he preferred to use phosphorylase from potatoes. We have repeated the work of Hanes and have tried phosphorylase preparations of various degrees of purity obtained from Laxton's Progress peas, jack beans, and potatoes. We agree with Hanes that potato phosphorylase is especially desirable. This is on account of the almost complete absence of amylase from preparations from potatoes; other plant materials often contain so much amylase as to interfere with the synthesis of glucose-1-phosphate. We have tested for amylase by inactivating the phosphorylase with formaldehyde, or by making extracts with 32 per cent acetone at 28°C., which destroys the phosphorylase. When potato phosphorylase solutions come in contact with air the oxidizing systems present soon destroy the phosphorylase, as Hanes found. Green and Stumpf (2) soaked potato slices in 0.005 M cyanide to prevent this destruction. We have ground our potatoes in dilute hydrocyanic acid solution for the same reason and have added still more hydrocyanic acid to our digests. We have not employed purified potato phosphorylase, since this enzyme, unlike jack bean phosphorylase, appears to be difficult to purify. Hanes used acid-washed kaolin to remove certain impurities from pea phosphorylase. We have found that some samples of acid-washed kaolin can be employed for this purpose, while other samples remove most of the phosphorylase.

Hanes concentrated his digests, prior to precipitating the barium salt of glucose-1-phosphate with alcohol, by freezing out about 80 per cent of the water. We have found this freezing out process to be extremely

tedious and consider it unnecessary. If this step is omitted one must use a rather large volume of alcohol, it is true, but the alcohol can be recovered by distillation. The glucose-1-phosphate isolated by us according to the procedure of Hanes is usually contaminated with about 40 per cent of impurities, among which are dextrins and potassium sulfate. In some preparations Hanes removed the dextrins by digesting the crude glucose-1-phosphate with purified α-malt amylase. We believe we have considerably improved the isolation procedure by introducing a preliminary digestion of the dextrins by pancreatic amylase. Instead of adding magnesium acetate and ammonia, by which Hanes removed phosphates, we have added barium acetate and ammonia.

The Method

Boil 8 g. of soluble starch with about 100 cc. of water. Cool and add 12 g. of Na_2HPO_4 (anhyd.) and 5 g. of KH_2PO_4 (anhyd.) dissolved in about 300 cc. of water. Add 100 cc. of potato-cyanide extract. Dilute to 1000 cc. Add toluene and mix. Keep at 20–25°C. for 24 hours.

Inactivate the phosphorylase by adding 0.1 N iodine solution until, upon mixing, the solution gives a permanent reddish-brown color. This destroys the phosphorylase. Now remove the iodine by adding 0.1 N sodium thiosulfate until the brown color is all gone. Add 10 to 20 cc. of 2 per cent pancreatin solution and allow the solution to stand at room temperature for 3 or 4 hours, or until a 1 cc. sample gives no test for dextrin when treated with 4 drops of 0.01 N iodine solution. Here it is best to compare with a blank containing water and 4 drops of iodine solution. Now add 40 g. of barium acetate and about 8 cc. of 28 per cent ammonia, or enough to make the solution decidedly alkaline to Phenol red. Mix well, centrifuge down the barium phosphate and filter the supernatant solution through cotton. Add to each volume of solution 2 volumes of 95 per cent alcohol. Mix well and centrifuge down the barium salt of glucose-1-phosphate. Discard the supernatant liquid. Stir the precipitate with 2 N sulfuric acid and 30 to 60 cc. of water. Add just enough sulfuric acid to give a pink color with Thymol blue paper. Now add saturated potassium hydroxide cautiously until the suspension just fails to give a blue or brown color with Congo red paper. Add 6 g. of trichloroacetic acid and mix. To every volume of the suspension add 2 volumes of 95 per cent alcohol and stir. Centrifuge down the suspended matter. Decant the clear supernatant solution. Add saturated potassium hydroxide until the solution is decidedly alkaline to Phenol red. The di-potassium salt of glucose-1-phosphate usually separates as an oil. Chill overnight at 5°C. or better at 0°C. Next day filter off the crystals of $C_6H_{11}O_9PK_2 \cdot 2H_2O$, wash several times with 95 per cent alcohol, then with acetone and dry at 40–50°C. The yield will be about 3.5 g.

The glucose-1-phosphate prepared as above will be about 85 per cent pure and will contain potassium sulfate. It can be obtained practically pure by recrystallizing once according to the procedure described by

Hanes. This consists in dissolving in 20 times its weight of distilled water, filtering clear, adding one volume of 95 per cent alcohol, mixing and chilling overnight. It is best to have the temperature not much higher than 0°C. The crystals of glucose-1-phosphate can then be filtered off and treated as described above. This material will have a specific rotation with sodium light of about +79° and a phosphorus content of about 8.33 per cent.

Preparation of Reagents

The potato-cyanide extract is prepared by disintegrating about 325 g. of potato (sliced a few seconds previously) in 100 cc. of 0.01 *N* hydrocyanic acid (KCN neutralized with hydrochloric acid) in a "Powermaster" blender. The disintegrated mass is pressed in cheesecloth, and the juice, amounting to about 175 cc., is centrifuged to free it from starch and cellular matter.

Thymol blue paper and Congo red paper are prepared by dipping filter paper in solutions of these dyes and then drying the paper.

Summary

A satisfactory and comparatively simple method for the preparation of glucose-1-phosphate has been described.

We wish to express our thanks to the Rockefeller Foundation for financial assistance.

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The Effect of α-Tocopherol and β-Carotene in the Oxidation of Plant and Animal Fats[1]

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Introduction

Since Olcott and Mattill (1) observed that antioxidants of plant origin were effective in stabilizing animal fats but had little or no effect on plant fats, it has been accepted generally that the mechanism of oxidation of these two fats is fundamentally different. Naturally, the question arose whether this difference was due to the nature of the glycerides or to the presence of contaminating substances. The findings of Swift, Rose, and Jamieson (2) that the induction period of methyl esters of the fatty acids from cottonseed oil was increased but little more by the addition of 0.05% tocopherol than by 0.025% tocopherol clearly implied that plant fats naturally contain so much antioxidant that further additions are ineffective. Golumbic (3) by a very skillful differential application of the Emmerie-Engel (4) and a modified Furter-Meyer (5) technique found that naturally occurring plant fats differ from animal fats in that they contain the precursors of chroman-5,6-quinones. As oxidation converts these precursors to the quinones—which are active antioxidants, they obscure the ending of the induction period. When the antioxidants were removed from plant fats by chromatographic adsorption the antioxidant effect of additions of tocopherol was easily demonstrable.

Both Swift and co-workers and Golumbic carried out their oxidations

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison.

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at 60–75°C. They furthermore measured the extent of oxidation and specifically the length of the induction periods mostly by peroxide titration or organoleptically. It seemed desirable that their work should be repeated using temperatures more nearly approximating those at which fats are usually kept or even temperatures approximating those of the animal body, since it is well known that peroxides are labile at higher temperatures. It also seemed desirable to determine the course of the oxidation by direct measurement of the oxygen absorbed. The results obtained, in so far as they are comparable, confirm those obtained by the aforementioned investigators. In addition, data on the pro-oxidant effect of β-carotene are presented.

Experimental

The fats used were lard, oleo oil, cottonseed oil, and soybean oil. The lard was rendered in the laboratory from fresh perinephritic tissue without contact with metal. The oleo oil was a fresh sample of Prime Oleo Oil[2] obtained commercially. It had been rendered at 68° C. then cooled in trays and separated from the crystalline glycerides by filtration with pressure. The cottonseed oil[3] and the soybean oil[4] were commercially refined samples. All fats were stored at −6° C.

In certain experiments ethyl linolate and ethyl oleate were used for comparison with the fats. The ethyl linolate was prepared by a procedure similar to that of Rollett (6) as modified by Quackenbush, Thompson, Cox, and Steenbock (7). The ethyl oleate was prepared from the fatty acids of olive oil by a method similar to that of Wheeler and Riemenschneider (8). The fatty acids were freed from most of the saturated acids by crystallization at −6° C. followed by recrystallization from acetone (10% solution) at −50° C. (Quackenbush and Steenbock, 9). The acid (I_2 No. 89.1) was esterified with EtOH in the presence of HCl. The ester was distilled through a Widmer column at 0.2–0.5 mm. Hg.

The rate of oxidation was measured with a Warburg respirometer by the following procedure: A petroleum ether solution containing the desired amount of antioxidant was pipetted into a 15 cc. flask. The solvent was removed under reduced pressure after which one gram of the fat was weighed or pipetted into the flask. The flask was connected to

[2] Armour and Company, Chicago, Illinois.
[3] Schieffelin and Company, New York, New York.
[4] Lever Brothers Company, Cambridge, Massachusetts.

the manometer assembly and shaken for 30 minutes at 37° C. to establish temperature equilibrium. The cock was then closed, and readings were taken at appropriate intervals. As the amount of oxygen absorbed was relatively large, mercury was used as the manometric fluid. A small drop of trimethylene glycol was added to each manometer to prevent adhesion of the mercury to the glass. The results were considered accurate to a unit reading on the manometer which corresponded to ± 0.025 cc.

The fats were freed from antioxidants by adsorption as follows: 200 cc. of a 10 per cent petroleum ether solution of the fat was forced through a column (20×180 mm.) of activated Al_2O_3 and then through a column of like dimensions containing 5 g. of Neutrol Filtrol[5] mixed with 10 g. Hy-Flo Super Cell[6]. The fats were finally freed from solvent by heating on a steam bath under reduced pressure.

The Effect of Tocopherol on Oxygen Absorption

To determine the effect of small amounts of *dl*-α-tocopherol, 4 γ were added to each sample of fat. This was approximately equal to the amount reported to be present in untreated lard (10), and about one per cent of that known to occur in cottonseed and soybean oils (11).

These additions were found to have no effect on the rate of oxidation, indicating that enough antioxidant was present in these natural fats of plant or animal origin to give them an induction period of at least 100 hours. In both instances, the plant fats absorbed more oxygen than the animal fats, *viz.*, 0.277 and 0.048 cc. per gram for lard and oleo oil and 0.532 and 0.363 cc. per gram for cottonseed and soybean oils, respectively.

To determine if small amounts of tocopherol had an effect on the same fats from which the antioxidants had been removed, 2 γ of tocopherol were added to the animal fats and 4 γ to the plant fats. A smaller amount of tocopherol was added to the animal fats because preliminary studies had shown that a given amount of tocopherol stabilized adsorbent-treated animal fats longer than plant fats.

The treatment with adsorbents reduced the induction periods of soybean and cottonseed oils from over 100 hours to 9 and 19 hours, respectively (Figs. 1 and 2). The induction periods of the lard and oleo oil were similarly reduced to 40 and 50 hours, respectively. The inhibiting effect of tocopherol was now demonstrable with both types of fats. Two

[5] Filtrol Corporation, Los Angeles, California.

[6] Johns-Manville Company, Chicago, Illinois.

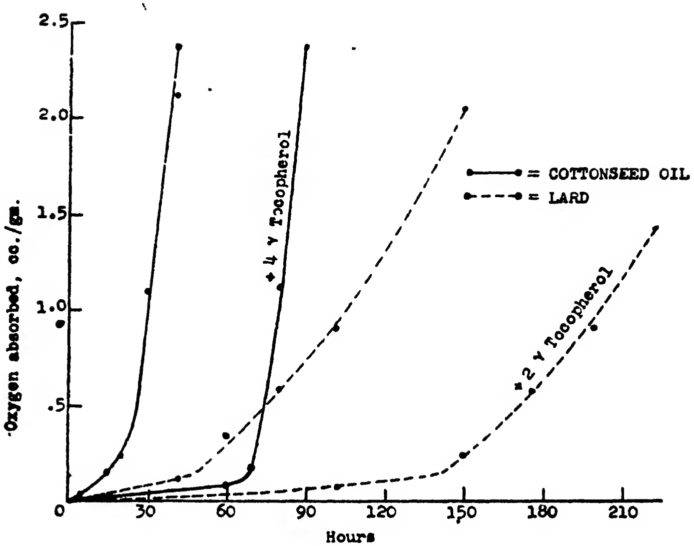

Fig. 1

The Effect of α-Tocopherol on Adsorbent-Treated Cottonseed Oil and Lard

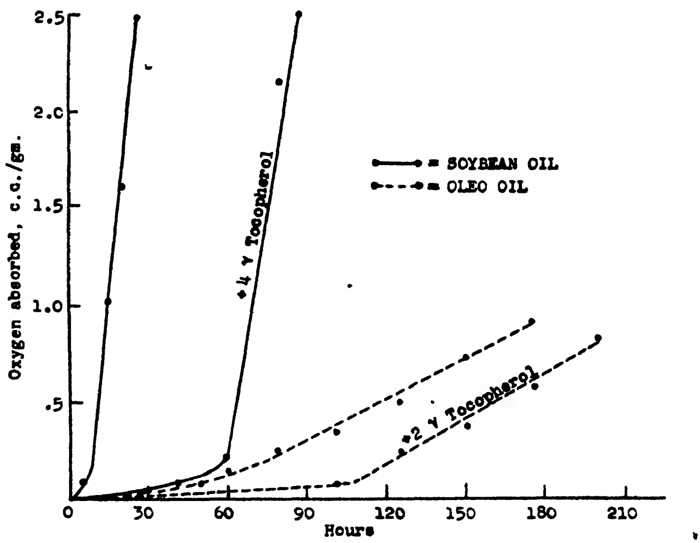

Fig. 2

The Effect of α-Tocopherol on Adsorbent-Treated Soybean Oil and Oleo Oil

TABLE I

The Effect of 250 γ β-Carotene per Gram on the Length of the Induction Periods of Adsorbent-Treated Animal and Plant Fats, With and Without Tocopherol Additions

Glyceride	Lard		Prime Oleo Oil		Cottonseed Oil		Soybean Oil	
Tocopherol added	0	2γ	0	2γ	0	4γ	0	4γ
No carotene (hours)	40	170	55	115	19	73	9	66
Plus carotene (hours)	0	38	0	42	2	4	0	6

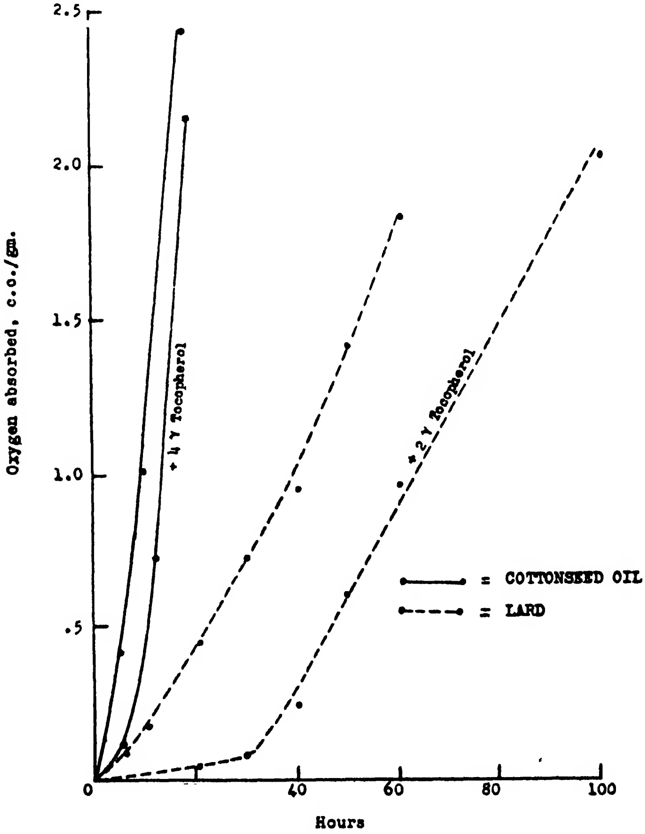

FIG. 3

The Effect of 250 γ β-Carotene per Gram on the Oxygen Absorption of Adsorbent-Treated Cottonseed Oil and Lard
(See Fig. 1 for controls without carotene.)

and 4 γ of tocopherol lengthened the induction periods from 50 to 110 hours.

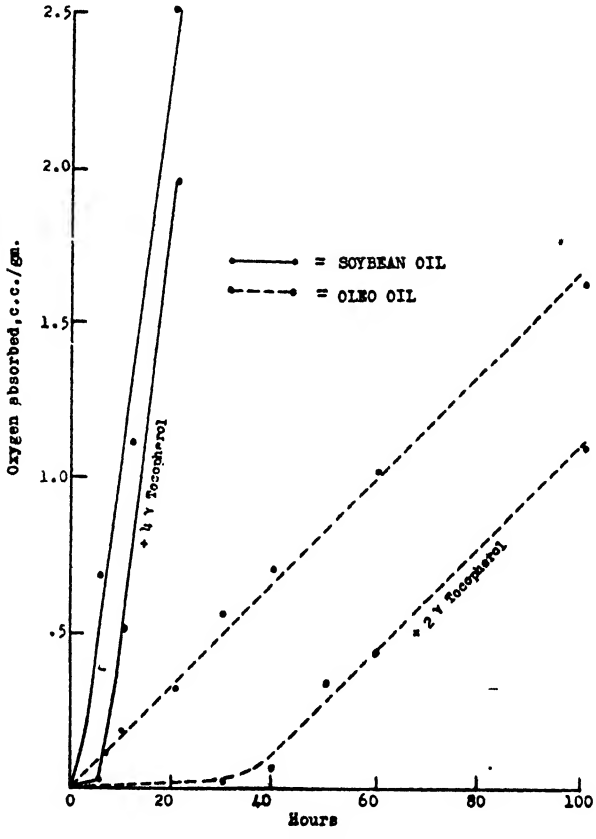

FIG. 4

The Effect of 250 γ β-Carotene per Gram on the Oxygen Absorption of Adsorbent-Treated Soybean Oil and Oleo Oil
(See Fig. 2 for controls without carotene.)

The Effect of β-Carotene on the Oxygen Absorption of Adsorbent-Treated Plant and Animal Fats

Further evidence of the similarity in the reactivity of plant and animal fats was obtained by the addition of a naturally occurring pro-oxidant, *viz.*, β-carotene. The carotene used was commercial β-carotene[7]

[7] Nutritional Research Associates, Incorporated, South Whitley, Indiana.

which had been purified by chromatographic adsorption on MgO and recrystallization from carbon disulfide. Two hundred-fifty γ were added to one gram samples of each of the four adsorbent-treated fats. In some cases small amounts of tocopherol were added in addition.

TABLE II

The Effect of Tocopherol on the Oxygen Absorption of Fatty Acid Esters in the Presence and Absence of 250 γ Carotene

Ester	Ethyl Oleate		Ethyl Linolate		Ethyl Oleate + Carotene		Ethyl Linolate + Carotene	
Tocopherol added	0	2γ	0	4γ	0	2γ	0	4γ
hours								
5	0	0	0.20	0	0.023	0	0.385	0.071
10	0	0	0.557	0.011	0.046	0.011	1.193	0.80
20	0	0	1.607	0.930	0.116	0.055	2.479	3.002
30	0	0	3.639	2.495	0.236	0.146	4.791	—
40	0	0	—	4.353	0.393	0.246		
80	0.036	0.012		—	0.777	0.618		
100	0.072	0.012			0.969	0.804		
150	0.116	0.067			1.396	1.282		
200	0.216	0.108			—	—		
250	0.356	0.221						
300	0.538	0.398						
Induction Period Hours	140	220	1	13	4	28	0	6

The results (Table I) show that carotene shortened the induction period of both plant and animal fats and accelerated the rate of oxidation in the period following the induction period (Figs. 3 and 4).

The Effect of Tocopherol and Carotene on Ethyl Linolate and Ethyl Oleate

As the plant fats were more unsaturated than the animal fats it was believed possible that the greater pro-oxidant effect of carotene on the former was due to their relatively greater degree of unsaturation. To test this theory, the rate of oxidation of two purified fatty esters, ethyl oleate and ethyl linolate, was determined under the conditions used in the preceding experiments. Two and 4 γ of tocopherol were added

respectively to one gram samples of ethyl oleate and ethyl linolate. Two hundred-fifty γ of carotene were added to the esters in another series of experiments.

The results (Table II) showed that these purified esters reacted similarly to the adsorbent-treated fats. Ethyl oleate had a long induction period (140 hours) followed by a period during which oxygen was absorbed slowly. Two γ of tocopherol prolonged the induction period to 220 hours. Ethyl linolate had a short induction period of one hour. This was increased to 13 hours by the addition of 4 γ of tocopherol; oxygen absorption then proceeded at the same rate as in the samples without any additions. Specifically, ethyl oleate reacted like the animal fats while ethyl linolate reacted like the plant fats.

The addition of carotene to the aforementioned esters also gave results similar to those observed with the fats; the length of the induction periods was shortened and the rate of oxidation following the induction periods was increased.

Summary

Small additions of α-tocopherol had no effect on the induction period of either plant or animal fats as represented respectively by cottonseed and soybean oil, and lard and oleo oil. However, after the fats had been freed from antioxidants by chromatographing, the antioxidant effect of such additions was easily demonstrable. These results, which were obtained by measuring the amount of oxygen absorbed at 37° C., confirmed the findings of others based on peroxide titration after aeration at 60–75° C.

β-Carotene was found to be an active pro-oxidant. It shortened the induction period and accelerated the rate of oxidation after the end of the induction period of both plant and animal fats from which the antioxidants had been removed chromatographically. The pro-oxidant effect of carotene was greater with plant fats than with animal fats. Similarly it was greater with ethyl linolate than with ethyl oleate. These analogies are in harmony with the relative degrees of unsaturation of these substrates.

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Enzymatic Decarboxylation of Oxalacetate and Carboxylation of Pyruvate*

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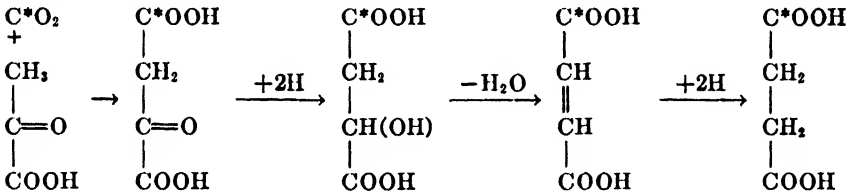
Introduction

Oxalacetate is an important intermediary in cellular metabolism and is the postulated intermediate in heterotrophic carbon dioxide assimilation. According to the mechanism first proposed for this reaction for the propionic acid bacteria (Wood and Werkman, 1940), pyruvate with carbon dioxide forms oxalacetate which is converted stepwise to malate, fumarate, and succinate:



Pyruvic acid   Oxalacetic acid   Malic acid   Fumaric acid   Succinic acid

*isotopic carbon, C^{13}

Various experimental observations definitely indicate that malate and fumarate are intermediates in the conversion of carbon dioxide and pyruvate to succinate. With the help of the isotope C^{13}, Wood, *et al.* (1940, 1941) presented evidence supporting this scheme by locating the fixed carbon dioxide in the carboxyl groups of succinic acid. Krebs and Eggleston (1941) showed that succinate is formed from pyruvate, oxalacetate, malate, and fumarate by the propionic acid bacteria, which was

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further evidence in support of the proposed scheme. Nishina, *et al.* (1941) using the C^{11} isotope, demonstrated the synthesis of malic and fumaric acids from pyruvic acid and carbon dioxide in fermentations by *Escherichia coli*, and Wood, *et al.* (1942) located the fixed carbon in the carboxyl groups of malate and fumarate during pyruvate dissimilation by pigeon liver, in the presence of malonate.

Evidence at present indicates that oxalacetate is a component of the fixation reaction (Werkman and Wood, 1942; Evans, *et al.*, 1943; Krebs, 1943). However, because of the instability and rapid dissimilation of oxalacetate, its formation from pyruvate and carbon dioxide has not yet been demonstrated. Block and Barron (*cf.* Barron, 1943) attempted to orient the metabolism of pyruvate toward the formation of oxalacetate by increasing the carbon dioxide tension and decreasing the temperature to 20°C., to avoid oxalacetate decomposition. Although there was vigorous pyruvate utilization in liver slices of rat and pigeon, no oxalacetate was detected. Krampitz, *et al.* (1943), using an acetone preparation of *Micrococcus lysodeikticus* could not demonstrate the formation of oxalacetate *via* a direct carboxylation of pyruvate. However, employing the enzyme preparation and the heavy carbon isotope, they did demonstrate that during the *decarboxylation* of oxalacetate to pyruvate and carbon dioxide, some *carboxylation* occurred. This was the first direct evidence that oxalacetic acid (or a closely related compound) is a component of the fixation reaction, and that the reaction

$$CO_2 + CH_3{\cdot}CO{\cdot}COOH \rightleftharpoons HOOC{\cdot}CH_2{\cdot}CO{\cdot}COOH$$

is reversible.

In the present investigation, an enzyme preparation obtained from *E. coli* has been employed to confirm and extend the work reported by previous investigators concerning the decarboxylation of oxalacetate. Quantitative data are also presented for the formation of oxalacetate (or a closely related compound) from succinate, fumarate, and malate. Finally the formation of "oxalacetate" directly from pyruvate and carbon dioxide has been demonstrated, *via* the Wood-Werkman fixation reaction.

Methods

The preparation of the enzyme system has already been described (Kalnitsky and Werkman, 1943).

Dialysis was carried out in a collodion bag against distilled water at approximately 5°C.

Oxalacetic acid was determined according to the methods of Edson (1935) and Straub (1936). Generally, the procedure followed in the formation and detection of oxalacetic acid according to Edson's aniline citrate method was as follows:

The enzyme plus buffer and water were placed in the main chamber of the Warburg cup, the substrate in one side arm, and 0.3 ml. of a 50 per cent solution of citric acid in the other side arm. After the substrate had been tipped into the main chamber, and the reaction allowed to proceed, the citric acid was also tipped into the main chamber; this procedure stopped the reaction and liberated all bound carbon dioxide. The manometers were shaken until all the bound carbon dioxide had been evolved (generally 5 to 15 minutes, depending on the amount of bicarbonate or carbon dioxide originally present). The manometers were then removed from the bath, 0.3 or 0.4 ml. of a 1:1 mixture of aniline and 50 per cent citric acid was added to the side arm originally containing the citric acid and the manometers quickly replaced on the bath. The contents of the cups were allowed to come to temperature equilibrium (3 to 5 minutes), readings taken, and the aniline citrate tipped into the main compartment of the cup. In the presence of oxalacetate, the carbon dioxide is completely evolved within 15 minutes, and originates from the carboxyl group adjacent to the methylene group of oxalacetic acid. The remainder of the oxalacetate molecule unites with the aniline to form pyruvanilide. Owing to the general instability of oxalacetate, especially at acid reactions, its determination must be carried out as quickly as possible. However, care must be taken that all the carbon dioxide is driven from the medium and the cup has reached temperature equilibrium before the aniline citrate is tipped into the main chamber. Controls are necessary, and if run correctly, give slightly negative or zero values for oxalacetate. If the aniline citrate is added after all the carbon dioxide has been liberated from the medium, the test can be adapted to detect small quantities of oxalacetic acid.

In determining oxalacetic acid colorimetrically, the procedure was as follows: The contents of the Warburg cup (2 ml.) were acidified with 0.3 ml. of a 10 per cent solution of trichloroacetic acid and quickly filtered through Whatman 42 filter paper, with a little suction applied to quickly filter off the proteins and to obtain as much of the original liquid as possible. Generally, about 1.8 to 1.9 ml. of liquid were obtained from the original contents of each cup. The liquid was filtered directly into a test tube containing 1.4 ml. of hydrazine reagent (3.5 g. of hydrazine hydrochloride dissolved in 30 ml. water, plus 100 ml. 95 per cent ethyl alcohol). The mixture was shaken, warmed for 15 minutes at 37° C., cooled in ice water for 3 minutes, 0.1 ml. of a saturated solution of $NaNO_2$ added, shaken, and allowed to stand for 5 minutes. Then 1 ml. of KOH solution was added (100 g. KOH plus 60 ml. water, the volume made up to 5 ml., and the yellow color read in a Klett-Summerson photoelectric colorimeter, with the 420 mμ filter. Known amounts of oxalacetic acid were plotted. Controls had a small blank reading, possibly due to the retention of some of the original color of the juice. Control values were subtracted from the experimental readings.

Pyruvate was determined colorimetrically according to the salicylaldehyde method of Straub (1936), as previously described (Kalnitsky and Werkman, 1943).

Experimental

Decarboxylation of Oxalacetate. The decarboxylation and the reduction of oxalacetate by many microorganisms and animal tissues have been reported by a number of investigators. The first bacterial β-decarboxylase to be described which brought about a rapid decarboxylation of oxalacetate was obtained from *Micrococcus lysodeikticus* by Krampitz and Werkman (1941). The enzyme was heat labile, and magnesium or manganese ions were found necessary for the reaction. Evans, *et al.* (1943), demonstrated that manganese ions functioned in the corresponding enzyme system of pigeon liver. In addition, the former group of investigators found that cocarboxylase did not function in the decarboxylation of oxalacetate. This work has been repeated, employing a dialyzed *E. coli* enzyme preparation, and substantially the same results were obtained. It was found that manganese is necessary for the reaction, whereas cocarboxylase has no effect. In addition, it was demonstrated that inorganic phosphate is not necessary for and does not accelerate the decarboxylation of oxalacetate.

There is a sharp dilution effect with the enzyme system concerned in the anaerobic dissimilation of pyruvate (Table I), but no such sharp effect was observed during the decarboxylation of oxalacetate (Table II). During the dissimilation of pyruvate, there was practically no activity with 0.5 ml. of the juice (80 μl. evolved), whereas with addition of 0.6 ml. of juice, the carbon dioxide evolution increased from 80 to 862 μl. Most of the carbon dioxide was evolved during the first hour and a half, but the experiment was allowed to proceed for three hours to determine whether there was an actual dilution effect or whether with smaller amounts of enzyme the rate of carbon dioxide evolution was just slower. Optimal decarboxylation of oxalacetate resulted with 0.7 ml. of the enzyme preparation (Table II) but even when as little as 0.1 or 0.2 ml. of the juice was used, definite activity was obtained.

Krebs (1942) has found that amino compounds, including amino acids, proteins, aniline, and multivalent ions also catalyze this decarboxylation. There is no doubt that Krampitz and Werkman (1941) demonstrated a heat labile enzyme which decarboxylated oxalacetate, and the preparation described here is quite similar. The specific protein nature of this enzyme was demonstrated by the precipitation of the proteins responsible for the decarboxylation by 50 per cent $(NH_4)_2SO_4$ (final concentration) (Table III), whereas the protein fraction precipitated with 45 per cent $(NH_4)_2SO_4$ had no decarboxylating activity on oxalace-

TABLE I

Effect of Enzyme Concentration on Dissimilation of Pyruvate

Enzyme $\mu l.$	CO_2 Evolved $\mu l.$
0.4	17
0.5	80
0.6	862
0.8	931
0.9	881
1.0	958

Each cup contained enzyme in indicated amounts; pyruvate, 0.035 M; $NaHCO_3$, 0.038 M; total volume, 2.0 ml.; atmosphere, CO_2; time, 3 hours; temperature, 30.4°C.

TABLE II

Effect of Enzyme Concentration on Decarboxylation of Oxalacetate

Enzyme $\mu l.$	CO_2 Evolved $\mu l.$
0.1	234
0.2	432
0.3	568
0.4	640
0.5	700
0.6	799
0.7	1110
0.8	1003

Each cup contained enzyme in indicated concentrations; oxalacetate, 0.045 M; phosphate buffer (pH 6.9), 0.05 M; citric acid (50%, in side arm), 0.3 ml.; total volume, 2.0 ml.; time, 0.5 hour; atmosphere, air; temperature, 30.4°C.; $\mu l.$ CO_2 equals CO_2 evolved, plus CO_2 evolved after acidification. Figures corrected for spontaneous decomposition of oxalacetate.

TABLE III

Fractional Precipitation of β-Decarboxylas

$(NH_4)_2SO_4$ %	CO_2 Evolved from Oxalacetate $\mu l.$
60	301
50	236
45	19
40	29

Each cup contained enzyme, 0.4 ml.; oxalacetate, 0.025 M; phosphate buffer (pH 6.9), 0.05 M; citric acid (in side arm, 50%) 0.3 ml.; total volume, 2.0 ml.; atmosphere, air; temperature, 30.4°C.; time, 0.5 hour. Figures corrected for spontaneous decarboxylation of oxalacetate. CO_2 evolved includes CO_2 liberated on acidification.

tate and did not increase the activity of the protein fraction precipitated by 50 per cent $(NH_4)_2SO_4$, on addition to it. Therefore, though proteins and amino acids may catalyze the spontaneous decarboxylation of oxalacetate, there is a specific protein system in this enzyme preparation which brings about a much more rapid decarboxylation. The activity of the β-decarboxylase seemed relatively unaffected by the pH of the medium, since oxalacetate was decarboxylated within a pH range of 2 to 10. The enzyme is saturated when the concentration of oxalacetate is approximately 0.03 to 0.05 *M* (Table IV), compared to approximately 0.03 *M* pyruvate concentration necessary for saturation during pyruvate dissimilation with the same preparation (Kalnitsky and Werkman, 1943).

TABLE IV

Concentration of Substrate and Saturation of Enzyme

Oxalacetate *M*	CO_2 Evolved $\mu l.$
0.01	206
0.03	300
0.04	313
0.05	384
0.06	375

Each cup contained enzyme, 0.3 ml.; oxalacetate in indicated concentrations; phosphate buffer (pH 6.9), 0.05 *M*; citric acid (in side arm, 50%) 0.3 ml.; total volume, 2.0 ml.; atmosphere, N_2; time, 0.5 hour; $\mu l.$ CO_2 includes CO_2 liberated on acidification. Corrections made for spontaneous decarboxylation of oxalacetate.

Formation of Oxalacetate from Succinate, Fumarate, and Malate. Banga (1936), used hydrazine as a fixative, to demonstrate the formation of oxalacetate from fumarate with washed muscle. Stare (1936) also showed the oxidative formation of oxalacetate from fumarate by liver and kidney tissues. Malic dehydrogenase from pig heart forms oxalacetate from *l*(-)malate in the presence of keto fixatives (Green, 1936). Among bacteria, the formation of oxalacetate from fumarate was demonstrated by Krampitz, *et al.* (1943); however no quantitative data were given.

Oxalacetate is formed from succinate, fumarate, and malate by the cell-free enzyme preparation obtained from *E. coli*. The presence of oxalacetate was determined by decarboxylation on addition of aniline

citrate (Edson, 1935). The action of the juice was stopped at the desired time by the addition of citric acid to the main chamber of the Warburg cup; thus any found carbon dioxide was released which might have been present. Oxalacetate is rapidly decarboxylated in the presence of aniline citrate, and so its presence can be determined quantitatively. Aniline citrate also slowly decarboxylates acetoacetate, but since this compound is not formed under the conditions of the experiment, the

TABLE V

Formation of Oxalacetate from Various Substrates by the Enzyme Preparation from E. coli

Substrate	O_2 Taken Up	CO_2 Evolved	Oxalacetate Formed
	µl.	*µl.*	*µl.*
Fumarate	−42	65	75
	−43	63	75
	−46	60	
	−41	66	
Fumarate Control	0	0	2
Malate	−25	70	
	−28	67	76
	−35	58	
Malate Control	0	0	−5
Succinate	−42	24	15
	−46	20	15
Succinate Control	0	0	1

Each cup contained enzyme, 0.8 ml.; substrate (fumarate and malate, 0.05 *M*; succinate, 0.045 *M*); citric acid (in side arm, 50 per cent), 0.3 ml.; total volume, 2.3 ml.; time, 1 hour; temperature, 30.4°C.; atmosphere, air; cups run in duplicate with NaOH in center well (0.3 ml. of 20% NaOH) to determine O_2 uptake and CO_2 evolution; 0.4 ml. aniline citrate added to determine oxalacetate.

aniline citrate test in this instance, is considered specific for oxalacetate. In all cases, controls were run. Aerobically, with 2688 µl. of fumarate or malate as substrate, about 75 µl. of oxalacetate were obtained at the end of one hour, with no fixatives or inhibitors present (Table V). Under the same conditions, much less oxalacetate is formed from succinate (approximately 15 µl.) The oxalacetate formed was completely destroyed by heating to 70 to 80°C. for 5 minutes to exhibit one of the

labile properties of ordinary oxalacetate. However, under an atmosphere of N_2, and with no O_2 present, no oxalacetate was formed from fumarate.

The fixation of carbon dioxide by this enzyme preparation and the location of the fixed carbon in the carboxyl groups of the succinic acid formed has been previously reported (Kalnitsky, *et al.*, 1943). Oxalacetate and fumarate are also reduced by this enzyme preparation (Kalnitsky and Werkman, 1943). These facts, plus the formation of oxalacetate from succinate, fumarate, and malate, demonstrate the possible and reversible occurrence of these intermediates during the utilization of carbon dioxide by this enzyme preparation.

TABLE VI

Absence of Oxalacetate Formation Under Optimal Conditions for CO_2-Utilization

Time *minutes*	CO_2 Evolved* *µl.*	Oxalacetate Formed *µl.*
10	+71	0
30	+104	0
45	+22	0
60	−47	−1
75	−117	0
180	−187	0

* + means CO_2 produced.
− means CO_2 missing, or fixed.

Each cup contained enzyme, 0.8 ml.; pyruvate, 0.026 *M*; $NaHCO_3$, 0.039 *M*; citric acid (in side arm, 50%), 0.3 ml.; total volume, 2.3 ml.; atmosphere, 5% CO_2 in H_2; temperature, 30.4° C.; 0.4 ml. aniline citrate added afterwards.

Carboxylation of Pyruvate. During the decarboxylation of oxalacetate by the enzyme, and in the presence of $NaHC^{13}O_3$, an exchange reaction took place, *i.e.*, an excess of heavy carbon was detected in the carboxyl group adjacent to the methylene carbon atom of the residual oxalacetate. This is an indirect demonstration of the occurrence of carboxylation, and is similar to and confirms the results previously obtained by Krampitz, *et al.* (1943). Since carbon dioxide fixation has already been demonstrated with this enzyme preparation with pyruvate as substrate, attempts were made to demonstrate direct carboxylation, by detecting the formation of oxalacetate from pyruvate and carbon dioxide.

Under optimal conditions for the formation of succinic acid and fixation of carbon dioxide by the juice, no test was obtained for oxalace-

tate (Table VI). An atmosphere of 5% CO_2 in H_2 was used, since under these conditions, the fixation of carbon dioxide and the formation of succinic acid was previously demonstrated (Kalnitsky, *et al.*, 1943). Duplicate control cups were acidified by simultaneously tipping the pyruvate and citric acid into the main chamber of the Warburg vessel containing the enzyme preparation and the bicarbonate. In the experimental cups the carbon dioxide evolved during the course of the fermentation, plus the carbon dioxide liberated on acidifying the contents of the Warburg vessels after the fermentation has proceeded for a definite length of time, equals the total carbon dioxide. The difference between the controls and the total carbon dioxide evolved from a cup where the fermentation was allowed to proceed, represents the carbon

TABLE VII

Formation of Oxalacetate from Pyruvate and CO_2

Atmosphere	Time *minutes*	Oxalacetate Formed *µl.*	Oxalacetate Formed (controls) *µl.*
5% CO_2 in N_2	25	22	−2
	60	24	0
	90	12	0
5% CO_2 in H_2	30	0	0
	90	0	0

Each cup contained enzyme, 0.8 ml.; pyruvate, 0.03 M; $NaHCO_3$, 0.045 M; citric acid (in side arm, 50%), 0.3 ml.; total volume, 2.3 ml.; temperature, 30.4°C.; aniline citrate added afterwards, 0.4 ml.

dioxide produced or utilized in the fermentation. It can be seen (Table VI) that after 30 minutes there was an excess of 104 µl. of carbon dioxide produced, but no oxalacetate was detected. After 45 minutes, the carbon dioxide excess was reduced to 22 µl. At 60 minutes, 47 µl. of carbon dioxide were missing, or fixed, but no oxalacetate was detected. After 75 minutes, and 3 hours, the amount of carbon dioxide fixed increased to 117 µl. and 187 µl. respectively, but in no case was oxalacetate detected. Any oxalacetate which might have been formed would almost immediately be reduced, in the presence of molecular hydrogen.

Under the same conditions, but substituting nitrogen for hydrogen to prevent the reduction of any oxalacetate a small but definite amount of oxalacetate was detected on the addition of aniline citrate to the medium after all bound carbon dioxide had been liberated (Table VII). The

exact procedure is described in the section on methods. The contents of the control cups were exactly the same as those of the experimental cups and were treated in the same way, except that the pyruvate was not tipped into the center well containing the juice until after the citric acid had been added. Again with the same juice under an atmosphere of 5% CO_2 in H_2, no test was obtained for oxalacetate.

In time-experiments conducted on the formation of oxalacetate from pyruvate and carbon dioxide, the amounts formed were generally slightly higher when the enzyme was allowed to act for 45 or 50 minutes. Smaller amounts of oxalacetate were detected at 20 minutes, and decreasing amounts were observed after a 60-minute period of activity. The amounts of oxalacetate formed were small, but each experiment was carried out at least in triplicate and repeated several times. The results checked well. Controls for each experiment were always run in duplicate under the exact conditions of the experimental cups and at the same time.

Effect of Enzyme and Substrate Concentration on Carboxylation. Definite effects were observed on varying the concentrations of the enzyme, pyruvate, and carbon dioxide. Table VIII shows the effect of varying the concentration of enzyme on the formation of oxalacetate from pyruvate. Here again, a dilution effect of the enzyme is observed since a decrease in the amount of juice from 0.8 to 0.4 ml. decreased the oxalacetate formed by two-thirds. Eight-tenths milliliter of juice was found to be optimal. On addition of 1.0 ml. of juice, and subsequent acidification in the Warburg cup longer shaking was necessary to completely liberate all the bound carbon dioxide, because of the greater quantity of precipitated proteins present. Because of the instability of oxalacetate some was probably lost during the longer shaking period. This may account for the somewhat erratic results obtained when larger amounts of enzyme were used.

The effect of pyruvate concentration on the formation of oxalacetate is shown in Table IX. It is evident that increasing the concentration of pyruvate results in quite small but definite increases in the quantity of oxalacetate formed. Carbon dioxide is also necessary for the reaction. During the anaerobic dissimilation of pyruvate a small amount of carbon dioxide is produced, and this amount is sufficient to cause the formation of some oxalacetate. However, when NaOH is added to the alkali well of the Warburg vessel during the dissimilation of pyruvate most of the CO_2 is taken up, and consequently the formation of oxalacetate is

negligible. The NaOH was removed before addition of aniline citrate to these cups. At a concentration of 0.018 *M* $NaHCO_3$, 20 μl. oxalacetate are formed. The optimal concentration of bicarbonate for the formation of oxalacetate is 0.035 *M*, at which concentration 29 to 30 μl. of oxalacetate are formed.

TABLE VIII

Effect of Concentration of Enzyme on Formation of Oxalacetate from Pyruvate

Enzyme	Oxalacetate Formed		
	Experimental	Average	Controls
ml.	*µl.*	*µl.*	*µl.*
0.4	8		0
	7	6	0
	3		
0.6	11		0
	14	12	0
	11		
0.8	21		0
	18	19	0
	18		
1.0	14		0
	27	19.5	0
	12		
	25		

Each cup contained enzyme in indicated amounts; pyruvate, 0.07 *M*; citric acid (in side arm, 50%, 0.3 ml.; total volume, 2.0 ml.; time, 50 minutes; atmosphere, 10% CO_2 in N_2; temperature, 30.4°C.; aniline citrate added afterwards, 0.4 ml.

In the presence of optimal concentrations of pyruvate, carbon dioxide, and the enzyme and in the absence of hydrogen gas or other suitable hydrogen donators, the presence of a small quantity of oxalacetate, or a compound very closely resembling it, can be detected.

Two mechanisms for the formation of succinic acid by this enzyme preparation have been demonstrated (Kalnitsky, *et al.*, 1943). Fumarate could conceivably arise from succinate formed by acetic acid condensation. However, under anaerobic conditions no oxalacetate was

detected with fumarate as substrate. There is a possibility that oxalacetate may be formed from fumarate anaerobically, if a suitable hydrogen acceptor such as pyruvate were present to alter conditions. It does not seem probable that the presence of a hydrogen acceptor such as pyruvate would alter conditions, because fumarate itself is a much better hydrogen acceptor than pyruvate. To further eliminate the possibility of oxalacetate formation from succinate which might arise by acetic acid condensation, KCN was used as a selective inhibitor. At concentrations of 0.0025 *M* to 0.005 *M* KCN, pyruvate dissimilation was inhibited 93% according to the carbon dioxide evolved from bicarbonate buffer, and 95% according to pyruvate actually dissimilated but the formation of oxalacetate from pyruvate was not inhibited. These results correspond with those of Wood and Werkman (1940) in work with the propionic

TABLE IX

Effect of Concentration of Pyruvate on Oxalacetate Formation

Pyruvate Concentration	Oxalacetate Formed		
	Experimental	Average	Controls
M	*µl.*	*µl.*	*µl.*
0.090	18 19 25	20.7	+1.5
0.070	17 24 21	20.7	−1.5 +1.5
0.053	14 14 14	14	+1.5 +4
0.035	11 10	10.5	0
0.018	0 0	0	0

Each cup contained enzyme, 0.8 ml.; pyruvate, in indicated concentrations; citric acid (50%, in side arm), 0.3 ml.; total volume, 2.0 ml.; aniline citrate added afterwards, 0.3 ml.; atmosphere, 10% CO_2 in N_2; time, 50 minutes; temperature, 30.4°C.

acid bacteria where it was found that KCN did not inhibit carbon dioxide fixation. Thus, the dissimilation of pyruvic acid to acetic and formic acids was almost completely stopped with no decrease in the amount of oxalacetate formed.

In attempts to further increase the carboxylation of pyruvate and the amounts of oxalacetate formed, KCN was of some value because it did not inhibit carbon dioxide utilization and it checked the spontaneous decarboxylation of oxalacetate. In the presence of cyanide, after 2.75 hours the amount of oxalacetate formed was increased to 36 μl.

TABLE X

Comparison of Aniline Citrate and Colorimetric Methods

Method	KCN	Oxalacetate Formed
	M	μ*l.*
Aniline citrate	0.00	16
	0.00	24
	0.0025	36
	0.0025	36
Colorimetric	0.005	31
	0.005	32
	0.005	37

Each cup contained enzyme, 0.8 ml.; pyruvate, 0.06 *M*; KCN, in indicated concentrations. In aniline citrate method, 0.3 ml. citric acid (50%) placed in side arm; 0.3 ml. aniline citrate added afterwards. In colorimetric method, 0.3 ml. of 10% trichloroacetic acid used as deproteinating agent. Total volume in each cup, 2.0 ml.; atmosphere, 10% CO_2 in N_2; time, 2.75 hours; temperature, 30.4° C.

(Table X). In the absence of cyanide, the amount was decreased considerably.

The Straub colorimetric test for the determination of oxalacetate (Straub, 1936) was also used. The presence of cyanide under the conditions employed did not interfere with the determination, and the amounts of oxalacetate formed, as determined by this method, checked well with the amounts of oxalacetate formed as determined with the aniline citrate method.

Two different methods have been employed to detect the formation of oxalacetate from pyruvate and carbon dioxide. The conclusive proof,

however, for the occurrence of this reaction would be the formation of oxalacetate using the tracer technique,

$$C^{13}O_2 + CH_3 \cdot CO \cdot COOH \rightleftharpoons HOOC^{13} \cdot CH_2 \cdot CO \cdot COOH$$

and locating the excess C^{13} in the carboxyl group adjacent to the methylene carbon atom of the oxalacetate formed. This has not yet been accomplished, because of the small quantities of oxalacetate detected and the general instability of the compound. Chemical analysis of the oxalacetate formed would also have to be made in order to determine whether the biological form is identical with the synthetic compound. The possibility remains that phosphorylated or other intermediates are involved in the fixation reaction, and that the reaction may be more complicated than represented.

Discussion

Assimilations or syntheses may generally be considered as endergonic biological reactions which require energy. To obtain this energy they are coupled with exergonic processes. Since the utilization of carbon dioxide is an assimilative reaction the energy for this assimilation may come from the splitting of some phosphate compound. According to Borsook (*cf.* Evans, *et al.* 1943), the decarboxylation of oxalacetate yields 5250 calories:

$$\begin{array}{ccccccc} \text{Oxalacetate} & + & H_2O & \longrightarrow & \text{Pyruvate} & + & HCO_3^- \\ -184{,}210 & & -56{,}200 & & -106{,}460 & & -139{,}200 \end{array}$$

$$\Delta F = -5250 \text{ Cal.}$$

The carboxylation of pyruvate would need that much energy in order to proceed. It is known that the formation and hydrolysis of pyrophosphate are two of the most energy-rich reactions in biological systems. The heat change (ΔH) in the splitting of pyrophosphate from adenosine-triphosphate amounts to about 11,000 Cal. per mole P. (*cf.* Kalckar, 1942). Thus the dephosphorylation of adenosinetriphosphate theoretially would supply enough energy for the carboxylation of two molecules of pyruvic acid.

Lipmann (1941) estimated the free energy change of the reaction phospho-*enol*-pyruvate → pyruvate + phosphate to be about —11,250 Cal. per mole P. Theoretically, then, this reaction could serve as a possible source of energy for the carboxylation reaction in living cells.

The formation of acetyl phosphate may be an important step indirectly connected with carboxylation. Lipmann established the oxidation of pyruvate through an intermediate phosphate to acetyl phosphate. Acetyl phosphate is quite unstable and forms acetic acid and inorganic phosphate at room temperature. In the presence of adenylic acid adenosine-triphosphate is formed (Lipmann, 1941), which provides its high energy of dephosphorylation to other chemical reactions. Confirmation of the occurrence of acetyl phosphate, this time under anaerobic conditions, during the dissimilation of pyruvate by an enzyme preparation of *E. coli* has recently been obtained by Utter and Werkman (1943). It was also demonstrated that acetyl phosphate transfers phosphate to adenylic acid, forming adenosine-triphosphate.

Thus, during the anaerobic dissimilation of pyruvate, two reactions in which we are interested, occur side by side. One, carboxylation, which needs energy; the other, the formation of acetyl phosphate, and then adenosine-triphosphate, the splitting of which yields energy. It is possible that these two reactions may be complementary.

Summary

Manganese is necessary for the decarboxylation of oxalacetate, whereas cocarboxylase and inorganic phosphate are not. The specific protein nature of the enzyme concerned in this reaction has been demonstrated.

The enzyme forms oxalacetate (or a compound closely related to it) from fumarate and malate, and smaller amounts from succinate aerobically. No oxalacetate is formed from fumarate anaerobically.

Under optimal conditions and in the absence of hydrogen or hydrogen donators, small amounts of oxalacetate (or a compound closely resembling it) are formed from pyruvate and carbon dioxide. Effects of concentration of enzyme, pyruvate, and carbon dioxide on the formation of "oxalacetate" have been studied.

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Crystalline Albumin from Chicken Blood[1]

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Blood was obtained from laying hens by cardiac puncture, according to the method of Sloan and Wilgus (1). Sodium citrate was used as the anticoagulant. The plasma from 150 200 ml. of blood was separated by centrifugation and was transferred to a dialyzing bag. It was adjusted to a pH of approximately 4.5 (brom cresol green) and then dialyzed in the refrigerator for at least 48 hours against distilled water to which a few drops of 1.0 N acetic acid were added. The precipitate, containing serum vitellin, fibrinogen, and probably part of the globulins (2) was centrifuged off and discarded. To the supernatant enough $M/1.5$ phosphate buffer of pH 7.2 was added to make final concentration $M/15$; then an equal volume of saturated $(NH_4)_2SO_4$ was added in order to precipitate the rest of the globulins which were filtered off and discarded. The filtrate was acidified with glacial acetic acid to pH 4.7, and left over night in the refrigerator. The precipitate was collected, dissolved in about 15 ml. of water, the pH of the solution was adjusted to 4.7, and saturated $(NH_4)_2SO_4$ was added drop by drop until an opalescence appeared. Usually even a very careful addition of $(NH_4)_2SO_4$ produced a rapid precipitation of amorphous protein. In such a case about 1 ml. of distilled water was added and the mixture was cooled down in the refrigerator, which resulted in the redissolving of the precipitate. Thereafter a drop of saturated $(NH_4)_2SO_4$ was added about every half hour until a faint precipitate appeared. The precipitate was examined under the microscope, and if amorphous it was filtered off and discarded. To the mother liquid a few more drops of saturated $(NH_4)_2SO_4$ were added until a new precipitate was formed.

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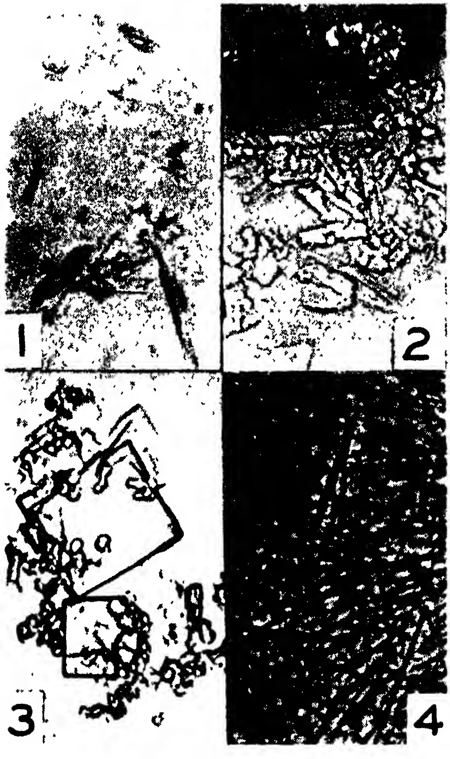
Usually it has been necessary to repeat this procedure 3 to 4 times before the precipitate showing crystalline form was obtained. From then on, saturated $(NH_4)_2SO_4$ was added 5 or 6 times a day a drop at a time. The crystallization was usually completed in 6 or 7 days.



Figs. 1-4

PLATE I

Crystalline Chicken Blood Albumin
Photomicrographs ×365

In the majority of cases crystals appeared as very thin, irregular, folded plates (Fig. 1). Rather often irregular but somewhat thicker plates were obtained, they were usually contaminated with spheroids (Fig. 2). In a few cases more regular and thicker plates were obtained, still slightly contaminated with spheroids (Fig. 3). Only once large, sharply defined needles were secured (Fig. 4), which after unsuccessful recrystallization changed again into the irregular plates.

Recrystallization was carried on essentially in the same manner as the first crystallization, except that the initial volume was usually smaller. Not much improvement in the crystalline form could be achieved, even after several recrystallizations.

The described method of fractionation of the plasma proteins showed a considerable difference existing between the blood of resting and laying hens. The first precipitate, consisting mainly of fibrinogen and serum vitellin, was much larger in the plasma of laying chickens. That was to be expected, it having been previously shown (3, 4) that no serum

TABLE I

Comparison of Serological Properties of Crystalline Blood and Egg Albumins

Effect of addition of 1 ml. of diluted rabbit anti-serum to 1 ml. portions of crystalline albumin solutions of various concentrations. Anti-serum was obtained from a rabbit immunized against crystalline egg albumin

	Protein content of 1 ml. of solution *mg.*	Anti-serum diluted 1:5 *ml.*	Reading after 2 hours of incubation
Crystalline blood albumin	2.0	1.0	–
	1.0	1.0	–
	0.5	1.0	–
	0.25	1.0	–
	0.125	1.0	–
	0.062	1.0	–
	0.031	1.0	–
	0.015	1.0	–
Crystalline egg albumin	1.0	1.0	Trace
	0.5	1.0	++
	0.25	1.0	+++
	0.125	1.0	++++
	0.062	1.0	+++
	0.031	1.0	++
	0.015	1.0	++

vitellin is present in the blood of resting birds. The second precipitate (mainly globulins) was larger in resting birds, and the third (albumin) was much smaller.

Of course, the described method of fractionation was entirely empirical. Since no evidence was secured that these fractions represented fractions of total globulin or total albumin, the quantitative determinations were deliberately omitted. One conclusion, however, seems safe enough; the amount of crystallizable albumin is larger in the blood of laying birds.

Crystalline serum albumin gives the same color reactions as crystalline egg albumin. It has the same general properties of solubility and apparently the same or very close isoelectric point.

The sample of crystals (shown in Fig. 4) after being recrystallized three times was examined serologically.[2] A rabbit was immunized with three times recrystallized egg albumin and its serum used for precipitin tests (Table I). Crystalline blood albumin was not serologically identical with egg albumin.

Summary

Albumin from the blood of laying hens was obtained in crystalline form. The chemical characteristics of crystalline egg and blood albumins were found to be very similar. Serologically the two albumins were not identical.

Indirect evidence was presented showing that the amount of albumin is increased in the blood of laying hens.

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[2] My thanks are due to Dr. M. H. Roepke of the University of Minnesota for the advice and help during this experiment.

Bound Water in Egg White

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Introduction

During a study of the problem of "watery" whites of eggs, determinations of bound water in the thick and thin portions of the white of hen eggs were made. St. John and Green (1930) studied the plasticity of the thick and thin portions of the white and presented definite evidence on the extent of the difference in mobility. They postulated a gross colloidal structure and obtained evidence of a time factor in the mobility of the thick white. On the basis of their paper and in view of considerations on the colloidal nature of egg white, of the large percentage of water in egg white, the lack of positive correlation of thin white content with wateriness (St. John, 1936), and the frequent association of a more mobile macroscopic appearance with the term "watery white," it was believed that a study of bound water in egg white might give pertinent information regarding its nature.

Although egg white is an easily obtainable natural biocolloidal material, few determinations of bound water have been reported. In addition to the data by St. John (1931), measurements have been reported by Newton and Martin (1930). The lower results which they report, however, may be due to the fact that the white was whipped before measurements were made, as suggested by St. John (1931) and endorsed by Jones and Gortner (1932) in their report of measurements on thick white by the dilatometer method. Caster and St. John (1944) reconsidered the formula for the calorimetric method and the results here presented are calculated in the light of these recent revisions.

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Experimental

The data reported in Table I were obtained by the method used by St. John (1931). Briefly, the method consisted of freezing a weighed amount of white at $-20°C$. and then melting it under conditions which permitted measurement of the heat absorbed. The weight of water frozen was calculated on the basis of the heat of fusion. Total moisture was determined in each sample and the bound (unfrozen) water calculated as percentage of total water.

Fresh eggs, not more than a few hours old, were used. They were classified, No. 1 being firm and No. 3 watery. Results on eggs which had been in storage from one to four months are included. A careful gravity separation of thick and thin white was made; these portions were transferred immediately with a minimum of manipulation to special separatory funnels. Bound water was determined on thick and thin white separately. A total of over 250 measurements were made. In much of the work duplicate determinations were made on both the thick and thin portions of the same egg.

TABLE I

Bound Water in Egg White

	Class 1		Class 2		Class 3		Classes 1, 2, 3 combined		Storage	
	Thin %	Thick %	Thin %	Thick %	Thin %	Thick %	Thin %	Thick %	Thin %	Thick %
Average	22.5	27.9	27.6	28.0	25.8	29.9	26.1	28.5	27.0	26.9
Maximum	38.7	40.3	38.7	42.2	41.7	36.1	41.7	42.2	45.8	43.6
Minimum	15.7	14.8	13.3	14.7	13.7	20.6	13.3	14.7	14.6	11.2
N	20	22	43	38	24	19	87	79	7	12
PE_s	3.89	3.64	4.88	3.26	5.9	3.28	5.15	3.38		
PE_m	0.85	0.71	0.63	0.45	1.06	0.65	0.49	0.33		

Discussion

In view of the probable deviation of the mean, the differences between thick and thin portions of the white in classes one (firm) and three (watery) would appear significant. However, the individual variation is large as shown by the probable deviation of a single determination. (A study of the cases where bound water was determined on the thick and the thin portions of the white of the same egg shows that in many individual instances the thin contained more bound water than the

thick.) This may be partly due to overlapping resulting from the individual variation. Seasonal variation may be suggested as a factor. There may also be a difference based on inherited characteristics as suggested by Lorenz, Taylor, and Almquist (1934).

Comparing the bound water in the different classes, the data do not definitely indicate a difference with regard to either thick or thin white. If there is any difference, it is a tendency toward more bound water in both portions of the more "watery" eggs. It was earlier assumed that "watery" eggs contained a larger proportion of thin white, but this apparent tendency toward increased bound water in "watery" eggs is in direct contradiction to the fact that there is a slightly larger amount of bound water in the thick white. These data, and those on storage eggs, thus question the postulation of Balls and Swenson (1934) that during storage, products of less hydrophilic type are formed, thereby liberating water which was previously bound to the mucin. These facts would again suggest, as did the data of St. John and Green (1930) and St. John (1936), that the difference between thick and thin white may not be the same as the difference between so-called "watery" eggs and firm eggs. A satisfactory explanation for a larger amount of bound water in watery eggs, while at the same time there appears to be a slightly larger amount of bound water in the thick portion of the white, is difficult to develop. It was postulated by St. John and Green (1930) that the thick white contained a colloidal structure which was largely, or at least probably partially, absent in the thin white. It is possible that the water binding capacity in the case of firm and watery eggs may be related to a difference in structural variation or size of colloidal particle. The more watery eggs may be composed of smaller colloidal particles, with greater surface which may result in opportunity for the binding of a greater quantity of water. On the other hand, the slightly larger amount of bound water in the thick white of the majority of eggs may be related to the relative amount of mucin in the thick and thin portions. Or it is possible that the relative water binding capacity is related to a difference in base exchange properties which may accompany the macroscopic differences evident.

The data for Classes 1, 2, and 3 were combined and averages and probable errors recalculated. These are also recorded in Table I. The probable error of the mean is of course reduced. On the basis of these averages and the probable error of their means, the difference between the thick and thin appears significant.

Bound water measurements were made on eggs which had been in storage from one to four months. The results are presented in Table I although the mean values are less reliable due to the smaller number of measurements. However, the results suggest that the bound water is approximately the same as in fresh eggs.

The results on bound water emphasize the variation between individual eggs. This variability has been emphasized by other papers from this laboratory based on other types of data, and also by other laboratories. The inheritance of albumen firmness has been discussed by Lorenz, Taylor, and Almquist (1934). The probable errors (Table I) indicate a greater variability in the thin than in the thick portion of the white. This greater variability may be related to a breaking-up of the thick portion to form an increased amount of thin white, and the resultant probable large variation in the size of the different portions or molecular aggregations of the thin white.

Methods used by Moran (1935) and Hill (1930) in preparing samples of egg white for bound water determinations might have a marked effect on the water-binding capacity on the albumin and perhaps a drastic effect in the case of recrystallized albumin. Hill and presumably Smith (1933) concentrated the egg white by evaporation. Moran used recrystallized albumin, and also concentrated by freezing. It should be expected that their results would vary from those of Jones and Gortner (1932) and St. John (1931) who made determinations without manipulation of the white taken directly from freshly broken eggs. Gortner's (1938, p. 301) statement regarding vapor pressure technique further explains the lower results obtained by Moran and by Hill. Kuhn (1924) suggested that a freezing method could measure only the intensity of water-binding rather than quantitatively measuring water-binding capacity. However, if this were true, the curve by St. John (1931) should continue to descend if the temperature is lowered below −12.5°C. The horizontal portion of this curve indicates an equilibrium and the measurement of a definite quantity. The effect of concentrating, refreezing, and recrystallizing should be determined.

Summary

As measured by the calorimetric method an average of approximately one fourth of the total moisture in fresh unmanipulated egg white exists in the "bound" condition. A large variation exists between individual eggs. The difference in the average per cent of bound water between

the thick and the thin portions of the white is not sufficiently large to be conclusive, although definite trends seem evident toward more bound water in watery eggs, and in contrast, toward a larger per cent in thick than in thin white. Variations in bound water do not correlate with the so-called "wateriness" of the white.

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A Study of the Heat of Fusion Method for Bound Water

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Introduction and Theory

In recent years, interest has developed in the physico-chemical condition of water in colloidal systems. Evidence of a considerable change in many of the properties of water in colloidal systems, and a study of the reasons for these changes have been largely responsible for the theory of bound, or unfree water.

The properties and theories advanced to explain water binding have suggested possible relationships between bound water and other phenomena. There may be some close association between bound water or the bound water-free water ratio and the adaptation of certain plants to drought or cold; fever in animals may in some way be related to bound water; bound water may be an index to certain metabolic responses in insects, or to the keeping quality of eggs.

Possible economic and academic application in such diverse fields before a large amount of basic information concerning water binding is at hand makes evident the importance of the study of the fundamentals underlying the bound water theory. Likewise, further investigation is needed before bound water can be concretely defined. Introduction of this physico-chemical concept has made necessary the development of methods, both qualitative and quantitative, of distinguishing between free and bound water.

Several methods have been used for the determination of bound water. Newton and Gortner (1922) introduced the cryoscopic method while studying hydrophilic colloids in plant tissue fluids. It is based on a determination of the freezing point and subsequent lowering of the

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freezing point by the addition of a weighed quantity of sugar. Another method, based on the expansion which occurs during the conversion of water into ice, was utilized by Foote and Saxton (1916), Bouyoucos (1917), Jones and Gortner (1932), and more recently by Buehrer and Rosenblum (1939) who refined the method by introducing evacuation technique. A third method uses the heat of fusion of ice as the basis for the calculation of bound water. This method appears to have been originated by Müller-Thurgau (1880). Kuhn (1924) and Sayre (1932) discussed its origin and history. It was given prominence by Rubner (1922) and was utilized by Thoenes (1925) and Robinson (1931). Thoenes developed a mathematical equation for the calculation of results. Improvements of the mathematical equation have since been developed by Robinson (1928) and St. John (1931). The equation has been further studied, somewhat more theoretically, by Sayre (1932) and Meyer (1932).

The purpose of the present paper is to discuss the advantages and disadvantages of the heat of fusion, or calorimetric, method for the measurement of bound water, and to evaluate the effect which each possible variable in the equation may have on the calculated results.

In the examination of the heat of fusion method, the equation developed by St. John (1931) was used with three minor changes, first, including a heat of fusion symbol, Q, as suggested by Meyer (1932); second, utilizing t to represent centigrade temperature; third, using S_i to represent specific heat of ice rather than using .5 as a constant. Sayre (1932) used a value for Q of 79.75. Thus the equation is as follows:

$$x = \frac{FN(t_3 - t_4) - (SW + S_1W_1)(t_4 - t_2)}{Q - S_i(t_2 - t_1)}$$

Definition of the symbols is stated below with the values used in the present study. These values were selected at random from a large number available on the thick portion of the white of fresh hen eggs. The procedure for the determination of bound water is discussed by St. John (1931).

Definition of the Symbols

Symbol	Value	Definition
F	1.1529	Correction factor (heat capacity) of calorimeter.
N	50. g.	Grams of water used in calorimeter.
W	13.0599 g.	Weight of sample used.

W_1	5.4672	Weight of lead crucible.
S	0.85	Specific heat of sample.
S_1	0.03	Specific heat of lead crucible.
S_i	0.5	Specific heat of ice.
t_1	−0.64° C.	Average freezing point of sample.
t_2	−20.2° C.	Temperature of sample in bath just before being transferred to calorimeter.
t_3	23.86° C.	Temperature of water in calorimeter before sample is added.
t_4	8.33° C.	Final temperature of calorimeter.
Q	80.	Heat of fusion of ice.
x	8.1720 g.	Grams of free (frozen) water.
y	11.0683 g.	Total water in sample.
z		Total solids in sample.

The specific heat of both free and bound water are taken as "one".

Substituting the values in the equation, we find the following:

$$x = \frac{1.1529 \times 50(23.86 - 8.33) - (0.85 \times 13.0599 + 0.03 \times 5.4672)(8.33 + 20.2)}{80 + 0.5(-20.2 + 0.64)} = 8.1720 \text{ g}$$

Then:

$$\text{Percentage of bound water} = 100\,\frac{(1 - 8.1720)}{(11.0683)} = 26.17$$

The effect of the variables will be considered individually, although it is apparent that a large number of combinations of effects might enter into the result. The effect of the deviation of the different variables is presented in Table I.

The data show that a variation in the value of S has a greater influence than any other measurement on the final bound water value.[1] The specific heat was considered to be 0.85 the value reported by St. John (1931). This value is subject to the limits of the graphs from which the temperatures t_3 and t_4 and the heat capacity factor, F, of the calorimeter are read. The effects of variations in S of ±0.02 and ±0.05 are reported in Table I. An accuracy of ±0.02 was required by Robinson; but a variation of ±0.05 is the more probable deviation in specific heat

[1] After this manuscript was submitted for publication the article by Freeman, *Arch. Biochem.* **1**, 27 (1943) came to our attention. Presumably it will be necessary to utilize correction factors for specific heat in the calculation of bound water in egg white and other lyophilic colloids.

determinations of materials other than egg white when using the undried sample for the determination.

TABLE I

Effect of Experimental Variations on Percentage Bound Water

Factor	Selected Variation	New value of x in g. (1)	Deviation in value of x in g.	New % bound water (2)	Deviation in value of % of bound water	% Deviation in bound water
F	0.001	8.1609	−0.0111	26.27	+0.10	0.38
N	0.16 g	8.1312	−0.0408	26.54	+0.37	1.41
W	0.0005 g	8.1718	−0.0002	26.17	±0.00	0.00
W_1	0.0005 g	8.1720	±0.0000	26.17	+0.00	0.00
S	0.02 cal	8.0658	−0.1062	27.13	+0.96	3.67
S	0.05 cal	7.9067	−0.2653	28.56	+2.39	9.13
S_1	0.0005 cal	8.1709	−0.0011	26.18	+0.01	0.04
S_1	0.013 cal	8.1430	−0.0290	26.43	+0.26	0.99
S_1W_1	(3)	8.2386	+0.0666	25.57	−0.60	2.29
$t_1:t_2$ const.	0.05°	8.1696	−0.0024	26.19	+0.02	0.08
$t_2:t_1$ const.	0.03°	8.1708	−0.0012	26.18	+0.01	0.04
t_2-t_1 (min.)	0.03°; 0.05°	8.1673	−0.0047	26.21	+0.04	0.15
t_2-t_1 (max.)	0.03°; 0.05°	8.1766	+0.0046	26.13	−0.04	0.15
$t_3:t_4$ const.	0.03°	8.1473	−0.0247	26.39	+0.22	0.84
t_3-t_4 (min.)	0.03°	8.1227	−0.0493	26.61	+0.44	1.68
t_3-t_4 (max.)	0.03°	8.2212	+0.0492	25.72	−0.45	1.72
$t_2:t_4$ const.	0.03°	8.1672	−0.0048	26.21	+0.04	0.15
t_4-t_2 (min.)	0.03	8.1816	+0.0096	26.08	−0.09	0.34
t_4-t_2 (max.)	0.03	8.1624	−0.0096	26.25	+0.08	0.31
Q	0.25 cal	8.2012	+0.0292	25.90	−0.27	1.03
Q	0.31 cal	8.2082	+0.0362	25.84	−0.33	1.26

(1) Original value of x = 8.1720 grams.
(2) Original per cent of bound water = 26.17.
(3) Values obtained if quantity S_1W_1 is omitted from equation.

The error introduced by not providing S_1W_1 to care for the effect due to the lead crucible is one of the most important in causing deviation. However, both S_1 and W_1 can be measured with precision.

The calorimeter temperatures, t_3 and t_4, are read from a graph which

is based on a series of readings of a thermometer which could be read to $\pm 0.05°$ C. Since the graph was made from a large number of points, and since it can be read to $\pm 0.02°$ C., its lower limit of accuracy is probably well within $\pm 0.03°$ C. The effect of variation in reading t_3 and t_4 are given showing the effect first if the error is made for one reading, the other being correctly read, and also the two possible cases when the error might be made in opposite directions, *i.e.*, giving maximum and also minimum values to $t_3 - t_4$. It appears that slight variations in these readings are third in the magnitude of their effects on the bound water value.

N is the number of grams of water used in the calorimeter. Calibration of the 50 ml. pipette at the experimental temperature of 23.86° C. showed the weight of the water to be 49.84 grams. This introduces a difference of 0.16 g. between the assigned value of the pipette and actual value of N. The effect of a variant value of 0.16 g. for N on the calculated amount of bound water as recorded in Table I places it in fourth place in magnitude of effect. Robinson (1927) observed that splashing of the calorimetric liquid was a serious source of error; however, he used only 10 ml. of water. Splashing can be avoided by careful manipulation. The relation between the size of the sample and the amount of liquid in the calorimeter is important since these two factors determine the magnitude of the temperature change, $t_3 - t_4$.

S_i and Q are constants whose values are not related to the particular sample under study. The specific heat of ice, S_i, has a value of 0.506 at 0° C., and 0.468 at −20° C. as found by Dickenson and Osborne (1915). The mean value between these limits is 0.487. The variation in bound water obtained by using this value is nearly 1% greater than that found by using the more common value of 0.5 for the specific heat of ice. The heat of fusion of ice, Q, has been given a value of 80 calories by all workers except Sayre (1932) who used 79.75. The value from Dickenson and Osborne's (1915) data is 79.69. The effect of deviating from the original 80 calories used by introducing the two lower values is shown to increase the bound water value by 1.03 and 1.26%, respectively.

Variation in the factors F, W, W_1, t_1, t_2, and S_1 have comparatively small influence on the final calculated percentage of bound water. F includes thermal effects of the calorimeter walls, the stirrer, stopper, and thermometer or thermopile. It was determined with the initial temperature at room temperature and can be evaluated on the calibration graph (St. John, 1931) to 0.0005.

The specific heat of the total solids, S_t, drops out in developing the equation and it is therefore unnecessary to measure it. The specific heat of both free and bound water are taken as "one".

To standardize the heat of fusion method for the measurement of bound water, some value for t_2 should be universally accepted. The value $-20°$ C. has been utilized by the majority of workers. The curve presented by St. John (1931) indicates that supercooling does not invalidate the use of $-20°$ C. for t_2. The method for the determination of total water in the sample should also be carefully standardized, since any variation in this value will materially affect the values of both amount and percentage of bound water. The authors evaporated overnight on a steam-bath and finally dried for an hour in a Freas oven at 100° C.

In all the single calculations reported in Table I, the error has been chosen so as to give the minimum amount of free water; hence, the maximum of bound water. This has been done because, as suggested by Gortner (1929), maximum results might be less desirable than minimum. Unwarranted significance might be attached to higher bound water values.

Discussion

It is apparent from the data in Table I that the factor defining the limits of accuracy of the bound water measurement is S, the specific heat of the sample. Using the limit of ± 0.02 suggested by Robinson (1931), a difference of 7.34% could result between duplicate samples if the 0.02 plus and minus values were obtained on duplicate samples. The values obtained using limits of ± 0.05 indicate an error so large that it seems imperative that S be determined by a method limiting the deviation to within even lower limits than Robinson suggested.

The temperature change which has the greatest effect in the equation is $t_3 - t_4$. If either a maximum or minimum value for $t_3 - t_4$ was chosen for one of two samples, an error of 2% could easily be introduced between duplicates by this one factor. The effect of ignoring the thermal capacity of the vessel containing the sample will materially decrease the calculated percentage of bound water. This value would be affected by the method of transferring the sample. The percentage of bound water obtained by using the more exact value for Q indicates that the value of 80 calories introduces an error which should be avoided. Variation in the values of W, W_1, S_1, t_1, and t_2 result in comparatively small changes in the percentage of bound water.

The features of the calorimetric method presenting the greatest difficulty are the determination of the freezing point and the specific heat of the material. The most satisfactory procedure in determining the freezing point is to graph readings against time intervals of fifteen seconds, a tedious process. Very seldom has a characteristic freezing point curve been obtained when using plant material, especially whole tissue. A change of slope must necessarily be taken as indicative of freezing. In the case of plant sap, this is frequently accompanied by an increase in viscosity which is noted as the bulb of the thermometer is used to agitate the sample. The measurement of electric current consumption to determine specific heat has been found advantageous in the case of sap. With whole tissues, the specific heat has been calculated from the number of calories of heat used in the calorimeter to warm the sample over a given temperature range.

Bound water has been expressed variously in terms of percentage of total water on a basis of (1) dry and of (2) green weight, and (3) as grams of bound water per gram of dry material in the sample. Any one of these methods might be satisfactory. It may, however, be advantageous to express results by all methods until sufficient data are available to indicate which is the most significant. The principal advantage of the heat of fusion method is that it may be used to study liquid, semiliquid, or solid material. Other methods are less adaptable.

Robinson (1931), expressing his results in grams of bound water per gram of dry material, reported a reproducibility of 1% using a gel. In biological tissue the variation was reported to be two to six times greater. Using Robinson's limits for S, Table I indicates that compensating errors probably enter in to give results of 1% agreement.

Each of the three general methods for the determination of bound water assumes no shift in the bound-free water ratio resulting from the freezing process, an assumption which may be incorrect. Kuhn (1924) expressed the belief that no freezing method would give a quantitative measure of water binding capacity but would measure only the intensity with which the water is bound. This is questioned by St. John and Caster (1944); however, it further emphasizes the importance of a standard temperature for freezing when using this method.

Summary

An examination of the heat of fusion method for the determination of bound water has been made to determine the effect of the accuracy of each individual measurement in the method on the accuracy of the final

calculated per cent of bound water. The measurement having the greatest influence is S, the specific heat of the sample; this value therefore requires the most care and accuracy in measurement. The factors, N, t_3, t_4, S_1W_1, S_i, and Q have an appreciable influence on the calculated percentage. Variation in the factors F, W, W_1, t_1, t_2, and S_1 have comparatively small influence on the final calculated percentage of bound water. N and W can be controlled to the necessary accuracy. S_i and Q are constant even though the absolute values may not be known. Three minor changes have been made in the equation by the utilization of Q for the heat of fusion of water, S_i for the specific heat of ice, and t in place of T. The principal disadvantage of the heat of fusion method is the necessity and the difficulty of determining freezing point and specific heat. The principal advantage in comparison with other methods for bound water is its wider applicability and adaptability.

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The Chemistry of Infectious Diseases:

VII. An Investigation of the Excretion of Certain Urinary Constituents during Type I Pneumococcal Pneumonia in Dogs

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Introduction

An increase in low molecular weight proteins (1-3) and a simultaneous decrease in albumin concentration (4) was found to occur in the serum of dogs with experimental lobar, Type I pneumococcal pneumonia. In normal dogs, and in dogs completely recovered from the infection, the peptone-like blood constituents are essentially absent from the serum, so that an efficient mechanism must exist by which they are removed during the course of the disease. One of the most likely pathways of elimination would seem through the kidneys, hence this investigation is a quantitative study of the urinary excretion of nitrogen, organic sulfur, inorganic and ethereal sulfates and thiosulfates throughout the entire course of the pneumonia. In addition, creatinine and creatine were determined; the former as a means of checking the completeness of urine collections during each of the daily test periods, and the latter for the purpose of having some means of differentiating between nitrogen eliminated due to tissue protein catabolism, and that derived directly from the serum proteins. Unfortunately data from creatine excretion can only be treated qualitatively since no data are available which permit of a quantitative correlation between the amounts of tissue protein destruction and creatine excretion, even when exogenous sources are excluded.

Sulfadiazine was given to both normal and infected dogs to determine

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whether or not sulfonamide therapy would influence the elimination of the above substances. Thiosulfate excretion was included, for it presumably arises in the intestines from the action of microorganisms on the ingested food. It appeared possible that sulfadiazine might inhibit the metabolism of these organisms.

Experimental

The dogs used throughout this investigation were thorough-bred bull terriers, 2 males and 4 females, kept in individual, metal metabolism cages. The former received 600 g. and the latter 500 g. of a 1:1 mixture of raw horse meat and Purina dog chow daily. The dogs had free access to water, provided in special containers to eliminate spillage. This quantity of food was consumed in a short time, but provided enough to insure maintenance of the initial body weights. Fasting periods for normal dogs were assumed to commence 24 hours after the last regular feeding period and lasted for 5 days. All animals were infected 24 hours after the last food intake. This regime of food consumption was adopted because it duplicated exactly for the normal fasting dog the conditions of the animals after infection. In this manner each dog served as his own control. After a fasting period, the dogs were maintained on a normal regime for about a month before they were infected. At that time a series of normal values was again secured.

Voided urine was collected under chloroform. In addition, each dog was catheterized daily at the same time each morning. The bladder was washed out twice with 50 to 100 cc. portions of water at 39° C. The urine and washings were acidified with 1–2 drops of concentrated HCl, then made to exactly 1,000 cc. with water, and filtered. During periods of sulfadiazine therapy, considerably more than 1,000 cc. of urine were excreted in 24 hours. This necessitated diluting the urine to the next higher 100 integer. Sulfadiazine was administered in 0.5 g. tablets, 2 g. of the drug every 4 hours, 3 times daily. The dogs were infected with Type I pneumococci as previously described (1).[2] The spread of the infection was followed by daily X-ray photographs.

Total nitrogen was determined by macro Kjeldahl, inorganic and total sulfates by Folin's method (5) and total sulfur by Denis' (6) modification of Benedict's (7) procedure. Creatine and creatinine were estimated with Folin's picric acid reagent (8), with the minor modification that the intensity of the color was read at 5200 Å in a 1-cm. cell with a Hardy spectrophotometer. At concentrations of 0.05 to 1.4 mg. of creatinine per aliquot of urine, the — log of the per cent transmission when plotted against creatinine concentration gave a straight line, and all readings were well reproducible when read 30 minutes after the addition of the alkali. Bratton and Marshall's (9) sulfanilamide method was used for the estimation of sulfadiazine in blood and urine, the violet color being read at 5450 Å in the spectrophotometer. In the case of urine the color intensity of the unknown had

[2] The preparation of the pneumococci cultures and the inoculation of the dogs was performed by Mr. Robert J. Fitzgerald to whom we wish to express our gratitude.

to be read about 1 minute after the addition of the *N*-(1-naphthyl)-ethylenediamine·2 HCl coupler. Substances other than sulfonamides appear to be present in dog's urine, which react slowly with the reagent.

Urinary thiosulfate was determined by Gast's method (10). Duplicate analyses of the same urine, made alkaline with NH_4OH in accordance with the procedure, checked each other well, but the recovery of added sodium thiosulfate was

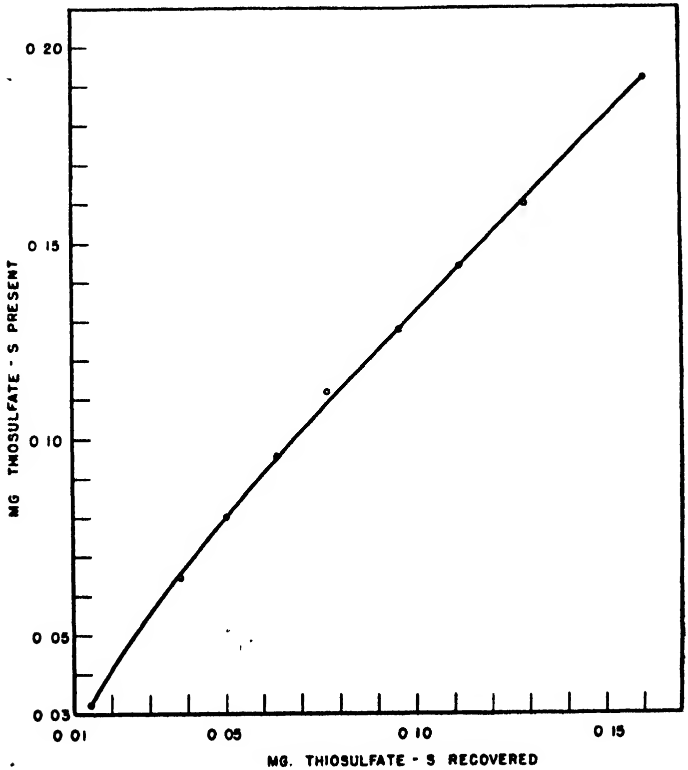

FIG. 1

Calibration Curve for Thiosulfate Determination

poor. It soon became apparent that the thiosulfate content of the female dog urine (from near zero to 0.2 mg. thiosulfate sulfur per 5 cc. of diluted urine) was outside the optimum range of the method. The percentage error decreased at higher thiosulfate concentrations, but rapidly increased in the lower ranges. When pure sodium thiosulfate solutions of known concentrations were analyzed and the amounts recovered were plotted against added sodium thiosulfate, a straight line was obtained in the range above 0.08 mg. Within the limits of experimental error, working at a range between 0.08 to 0.16 mg. thiosulfate-sulfur

per 5 cc. of standard solution, a constant loss of 0.0314–0.0349 mg. occurred, and it was felt that perhaps this value represents the solubility of the nickel ethylene diamine thiosulfate in 12–15 cc. of water-alcohol mixture. Below a concentration of 0.08 mg. recoveries were too erratic to make them usable, due to greater losses. For this reason, whenever urine samples were found to contain less than 0.08 mg. thiosulfate-sulfur per 5 cc. of urine, the analyses were repeated with 0.1 to 0.15 cc. of exactly 0.01 N $Na_2S_2O_3$ added to 5 cc. of urine. This brought the titration values into that part of the curve (Fig. 1) where the graph was a straight line.

All analyses were made at least in duplicate, and if occasionally the agreement was poor, a third one was performed.

Results and Discussion

One of the 5 dogs inoculated was apparently immune to Type I pneumococci. Two inoculations, the second after a 4 months' interval, failed to produce an infection. Most of the data on this dog are, therefore, omitted from the tables. The infection proved fatal to 2 of the remaining 4 dogs. The 2 animals which recovered were reinfected after several months' rest periods, but only one of these reinfections developed into typical pneumonia.

Normal Dogs

Nitrogen, creatinine, and the various sulfur compounds for which analyses were made were excreted by the normal dogs at almost constant amounts during every 24-hour period. However, there was a relatively large daily variation in creatine excretion even though the food intake was kept constant. These data are summarized in Tables I–VII.

As shown in Tables I–IV, a decrease of about 50% in nitrogen, organic-sulfur, inorganic- and ethereal-sulfate excretion occurred during periods of fasting. After 48 hours, a relatively constant minimum level was reached for each of these urinary constituents which remained unchanged until food was again consumed. Following the termination of the fast after the 5th day, normal levels were again attained within 48 hours. Very little creatine was excreted during fasting periods; the values reported in Table VI are almost identical with those published by Howe, *et al.* (11) on fasting dogs. Sulfadiazine administration during fasting periods had no influence on the low level of creatine excretion. The daily output of urinary creatinine remained unaffected by fasting, or by sulfadiazine administered to normal and to fasting dogs. This is in accordance with Folin's (12) original observation, since then amply confirmed, that urinary creatinine is of endogenous origin. Since neither sulfadiazine nor a severe pneumonia had any influence on the urinary

creatinine level, its elimination was used as a criterion of the completeness of urine collection.

The complete absence of thiosulfates during periods of fast, and their prompt reappearance after feeding is resumed suggests that urinary

TABLE I

Daily Urinary Nitrogen Excretion of Normal and of Pneumococcus Infected Dogs

(g. of Nitrogen per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male	
Normal Min.-Max. Values	10.68–13.25	11.18–12.39	12.32–14.51	10.01–13.69	9.36–11.10	9.79–13.72	11.07–13.76	10.84–13.67	12.57–13.41
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine
Successive 24-Hour Periods	4.55	6.10	6.82*	5.81	6.24	6.52	5.20	6.58	7.86
	3.89	9.40	9.78	4.09	7.70	4.59	7.62	4.57	8.54
	3.81	10.40	11.38	4.11	9.80	4.94	6.60	4.04	7.95
				4.05	12.38	4.36	died		
				4.14	died	4.15			
	Normal Treatment Resumed								
	7.76	16.72	15.75**	10.44		10.92		9.68	16.14**
	10.12	16.10	13.40	11.84		10.54		13.00	15.54†
	12.66	15.28	15.12†	11.48		11.94		13.65	16.23
	11.45	17.16	14.64			10.66			12.68
		14.42	14.34						14.02
		14.11	normal						13.75
		13.47							normal
		13.34							
		normal							

* Drug therapy not begun until second day.

** Drug therapy continued.

† Drug discontinued.

thiosulfates arise from the action of the intestinal flora. Sulfadiazine administration did not influence the level of thiosulfate excretion. If it is correct that urinary thiosulfate is a metabolic product of the intestinal microorganisms (13, 14), then it would appear that sulfadiazine has

either no inhibitory action on these microbes, or else the drug is absorbed too quickly from the intestines to be able to exert its inhibitory action on the microorganisms responsible for thiosulfate production. It would be

TABLE II

Daily Urinary Inorganic Sulfate Excretion of Normal and of Pneumococcus Infected Dogs

(g. of Inorganic SO_4-S per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male	
Normal Min.-Max. Values	0.46–0.59	0.48–0.56	0.48–0.58	0.38–0.56	0.27–0.36	0.43–0.62	0.43–0.59	0.33–0.41	0.44–0.49
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine
Successive 24-Hour Periods	0.19	0.45	0.35*	0.24	0.32	0.25	0.29	0.20	0.37
	0.15	0.41	0.33	0.15	0.31	0.21	0.32	0.15	0.24
	0.16	0.60	0.57	0.16	0.43	0.22	0.27	0.13	0.36
				0.16	0.60	0.21	died		
				0.14	died	0.20			
	Normal Treatment Resumed								
	0.30	0.75	0.72**	0.32		0.47		0.27	0.62**
	0.42	0.80	0.57	0.37		0.44		0.34	0.57†
	0.51	0.80	0.56†	0.36		0.45		0.36	0.63
	0.42	0.79	0.57			0.44			0.50
		0.65	0.62						0.57
		0.63	0.61						0.54
		0.56	normal						normal
		normal							

* Drug therapy not begun until second day.
** Drug therapy continued.
† Drug discontinued.

of interest to investigate the action of a poorly absorbed sulfonamide, such as sulfaguanidine, on thiosulfate excretion. The 4 female dogs excreted normally between 2 and 15 mg. of thiosulfate-sulfur per 24 hours, while the urine of the males contained during the same time intervals from 50 to 125 mg. Whether this observation was fortuitous—

and we can hardly believe that such was the case here—or whether the intestinal tract of female bull terriers differed in some unknown respect to make microbic thiosulfate formation more difficult, is not known. However, the withholding of food stopped thiosulfate excretion in the

TABLE III

Daily Urinary Organic Sulfur Excretion of Normal and of Pneumococcus Infected Dogs

(g. of Organic-S per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male	
Normal Min.-Max. Values	0.08–0.10	0.08–0.18	0.13–0.17	0.08–0.13	0.09–0.10	0.07–0.10	0.12–0.18	0.18–0.28	0.21–0.23
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine
Successive 24-Hour Periods	0.04	0.15	0.27*	0.13	0.25	0.06	0.10	0.19	0.30
	0.05	0.23	0.18	0.15	0.21	0.10	0.25	0.15	0.31
	0.05	0.11	0.27	0.12	0.25	0.06	0.27	0.14	0.16
				0.13	0.32	0.06	died		
				0.12	died	0.05			
	Normal Treatment Resumed								
	0.05	0.11	0.24**	0.16		0.08		0.26	0.26**
	0.07	0.14	0.28	0.13		0.07		0.22	0.27†
	0.09	0.19	0.26†	0.13		0.07		0.21	0.22
	0.09	0.09	0.26			0.08			0.14
		normal	0.19						0.14
			0.11						normal
			normal						

* Drug therapy not begun until second day.

** Drug therapy continued.

† Drug discontinued.

male dogs as completely as in the females. Because of this observation data on thiosulfate excretion of the normal male, Dog 58 was included in Table V; otherwise this animal is omitted from the discussion due to his accidental death prior to infection.

Since the amounts of urinary constituents excreted by normal, not

fasting dogs, were found to be the same in the absence or presence of sulfadiazine, these data have been omitted.

No acetylated drug was found in the urine of normal, fasting, or infected dogs. This is in agreement with the reported observations of

TABLE IV

Daily Urinary Ethereal Sulfate Excretion of Normal and of Pneumococcus Infected Dogs

(g. of Ethereal SO_4-S per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male	
Normal Min.-Max. Values	0.04–0.05	0.03–0.04	0.03–0.04	0.03–0.04	0.02–0.04	0.02–0.04	0.02–0.04	0.04–0.05	0.03–0.04
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine
Successive 24-Hour Periods	0.02	0.03	0.02*	0.03	0.02	0.02	0.02	0.02	0.02
	0.02	0.02	0.03	0.02	0.02	0.01	0.03	0.02	0.01
	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02
				0.01	0.02	0.01	died		
				0.02	died	0.01			
	Normal Treatment Resumed								
	0.03	0.04	0.03**	0.03		0.04		0.05	0.04**
	0.04	0.02	0.04	0.03		0.04		0.04	0.05†
	0.03	0.03	0.04†	0.03		0.03		0.05	0.04
	0.05	0.04	0.04			0.03			0.02
		0.05	0.04						0.03
		0.04	0.04						normal
		normal	normal						

* Drug therapy not begun until second day.

** Drug therapy continued.

† Drug discontinued.

other investigators (15) that dogs differ from man, rabbit, rat, mouse, and monkey in that they are incapable of acetylating the sulfonamides. The administration of sulfadiazine caused considerable diuresis, both in normal and in fasting dogs. In Table VIII data are presented on the daily urine excretion of normal and of fasting dogs during sulfadiazine

therapy while Table IX presents similar data on infected animals. The administration of 6 g. of sulfadiazine daily for 4 or 5 days to dogs weigh-

TABLE V

Daily Urinary Thiosulfate Excretion of Normal and of Pneumococcal Infected Dogs

(mg. of Thiosulfate-S per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male		Dog 58 Male
Normal Min.-Max. Values	6.6–7.6	4.6–6.0	5.8–9.8	2.2–14.2	5.2–7.6	5.6–8.2	5.2–8.8	51.8–123.2	62.0–88.0	47.0–94.9
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine	Fasted; S-diazine
Successive 24-Hour Periods	none	7.4	8.0*	none	3.4	1.8	7.8	none	44.5	none
	none	none	none	none	4.2	0.2	7.8	none	none	none
	none	10.4	8.6	none	5.2	1.6	6.6	none	5.2	none
				none	9.6	none	died			none
				none	died	none				none
	Normal Treatment Resumed									
	6.0	20.8	12.3**	none		6.8		15.2	29.0**	26.4
	10.6	none	12.4	none		none		46.0	32.3†	43.6
	8.6	none	10.0†			5.8		71.7	49.5	58.8
	7.2	13.6	14.0			5.8			32.5	59.1
		9.5	13.6						33.5	63.9
		9.4	12.0						41.5	
		8.0	9.4							
		8.4	normal							
		7.2								
		normal								

* Drug therapy not begun until second day.

** Drug therapy continued.

† Drug discontinued.

ing approximately 35 lbs. increased the volume of urine from below 600 cc. to above 1500 cc. One even reached 2300 cc. Although practically no drug was excreted beginning with the 4th day after drug therapy

stopped, several more days elapsed before the urine volume returned to normal. Only a fraction of the total, ingested sulfonamide was recovered from the urines in every instance. When normal dogs were

TABLE VI

Daily Urinary Creatine Excretion of Normal and of Pneumococcus Infected Dogs

(g. of Creatine per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male	
Normal Min.-Max.Values	0.48–0.61	0.28–0.46	0.43–0.61	0.16–0.69	0.15–0.35	0.17–0.37	0.42–0.63	0.33–0.77	0.36–0.57
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine
Successive 24-Hour Periods	0.09	0.17	0.23*	0.11	0.10	0.02	0.26	0.16	0.26
	0.06	0.88	0.99	0.08	0.80	0.03	0.79	0.09	0.88
	0.01	0.77	0.79	0.06	0.85	0.01	0.88	0.06	0.33
				0.04	0.89	0.04	died		
				0.05	died	0.06			
	Normal Treatment Resumed								
	0.20	1.02	1.02**	0.21		0.23		0.42	0.96**
	0.11	0.71	0.25	0.22		0.11		0.36	0.25†
	0.30	0.91	0.16†	0.21		0.15		0.40	0.20
	0.14	0.69	0.11			0.23			0.09
		0.40	0.07						0.10
		0.32	0.12						0.21
		0.25							0.31
		0.24							normal
		normal							

* Drug therapy not begun until second day.

** Drug therapy continued.

† Drug discontinued.

given sulfadiazine during fasting periods, diuresis did not occur until the dog was fed again, and it thus appears that sulfonamide therapy produces diuresis only when food is available. It seems unlikely that fasting dogs drink less water during drug therapy. Dogs not receiving

drugs excrete during fasting periods normal quantities of urine. In the case of the infected animals, the data are somewhat inconclusive, as shown in Table IX. The results indicate that those animals which were

TABLE VII

Daily Urinary Creatinine Excretion of Normal and of Pneumococcus Infected Dogs

(g. of Creatinine per 24-Hour Period)

	Dog 55 Female			Dog 56 Female		Dog 53 Female		Dog 57 Male	
Normal Min.-Max. Values	0.64–0.78	0.63–0.67	0.56–0.66	0.52–0.67	0.53–0.62	0.69–0.88	0.45–0.61	0.57–0.68	0.49–0.59
	Fasted	Fasted; Infected	Fasted; Infected; S-diazine	Fasted; S-diazine	Fasted; Infected	Fasted	Fasted; Infected	Fasted; S-diazine	Fasted; Infected; S-diazine
Successive 24-Hour Periods	0.64	0.73	0.69*	0.57	0.61	0.75	0.36	0.67	0.61
	0.66	0.84	0.81	0.53	0.66	0.65	0.59	0.61	0.47
	0.73	0.71	0.74	0.54	0.53	0.71	0.45	0.54	0.55
				0.56	0.40	0.67	died		
				0.55	died	0.65			
	Normal Treatment Resumed								
	0.66	0.77	0.62**	0.53		0.82		?	0.58**
	0.66	0.35	0.50	0.57		0.69		0.48	0.57†
	0.63	0.48	0.46†	0.60		0.75		0.60	0.57
	0.65	0.50	0.56			0.76			0.45
		0.56	0.57						0.49
		0.53	normal						0.50
		0.56							normal
		0.58							
		normal							

* Drug therapy not begun until second day.
** Drug therapy continued.
† Drug discontinued.

very ill and eventually succumbed to the infection, showed no diuresis while the other 3 dogs, which survived, behaved like the normal animals. From information gained during previous studies on dogs with pneumococcal infections, it is pretty certain that during the febrile stages of the disease, dogs consume more water than normally but do not lose it by

increased urine elimination. Animals when very ill will exert themselves as little as possible for one or more days prior to death and may in such circumstances then consume less water. The volume of urine ex-

TABLE VIII

Diuresis Produced in Normal Dogs as a Result of Sulfadiazine Therapy

(ml. Urine)

	Dog 52 Female	Dog 53 Female	Dog 55 Female	Dog 52 Female	Dog 53 Female	Dog 55 Female	Dog 56 Female	Dog 57 Male	Dog 58 Male
Normal Volume of Urine	*1000	*1000	<600	<600	<600	<600	<600	<600	<600
	Sulfadiazine to Non-fasting Dogs			Sulfadiazine to Fasting Dogs					
	2000	1000	390	<600	<600	<600	480	380	<600
	2000	1500	1915	<600	<600	<600	750	480	<600
	2300	1500	2000	<600	<600	<600	610	550	<600
	2100	1500	1710	<600	<600	<600	690	565	<600
			2070	<600			540	700	980
Successive 24-Hour Periods	Sulfadiazine Discontinued			Dogs Fed, Sulfadiazine Discontinued					
	2000	1500	1990	2000	1300	1960	1510	1640	1630
	2000	1100	1510	2000	1390	1680	1290	1390	1870
	2000	1200	1985	2000	?	1640	1110	1110	1500
	2000	1000	1785	1350	?	1400	<600	<600	1200
	1500		1685	<600	1020	1120			<600
	1350		1280		<600	<600			
	1150		1165						
	1300								
	1055								
	1000								

* Volumes in this column represent urine collection after dilution.

creted by dogs less critically ill, as, for instance, Dogs 55 and 57, was approximately equal to that of normal dogs.

Infected Dogs

Since, as shown above, the urinary excretions of nitrogen and of the various sulfur compounds decreased during fasting periods, and since

dogs will not accept food during the first 3 or more days after infection, it became necessary to use normal dogs as controls for the infected animals and to subject the former to fasting periods of the same duration.

TABLE IX

Diuresis Produced as a Result of Sulfadiazine Therapy in Dogs During Pneumonia

(ml. Urine)

	Dog 55 Female	Dog 56 Female	Dog 53 Female	Dog 55 Female	Dog 57 Male
Normal Volume of Urine	<600	<600	<600	<600	<600
	Dogs Infected and Fasted No Drug Therapy		Sulfadiazine to Fasted, Infected Dogs		
Successive 24-Hour Periods	900	930	200	660	320
	1400	600	440	820	300
	1730	600	400	1000	300
		550	died		
		died			
	Dogs Fed				
	1530			1230	540*
	1880			1370	1270
	2250			1700*	1040
	1410			1640	740
	780			1550	700
	830			1420	600
	600			1060	<600
	600			960	
	430			670	
	<600			650	
				480	
				<600	

* Drug stopped.

Hence, the data in Tables I–VII of infected, voluntarily fasting dogs are arranged for each 24-hour period side by side with data obtained from the same dog prior to the infection but during involuntary fasting periods. This makes it possible to compare readily the quantities of urinary constituents excreted daily by the same dog before and after

infection under otherwise identical conditions. Thus, the data for the nitrogen excretion of Dog 55 in Table I show that the amount of nitrogen excreted by this animal prior to fasting varied between 10.7 and 13.3 g. per 24-hour period, and that approximately one month later the same animal prior to infection excreted daily the same amount of nitrogen, namely between 11.2 and 12.4 g. After infection the dog refused food for 3 days. The quantities of nitrogen excreted during 3 days by the normal, fasting dog (1st Column of Table I, Dog 55) were 4.55, 3.89, and 3.81 g. during the 1st, 2nd, and 3rd days of fasting, while the infected animal excreted 6.10, 9.40, and 10.40 g., respectively, (2nd Column of Table I, Dog 55) for the same periods when no food was consumed. Food offered to the infected dog on the 4th day was almost completely consumed. Hence the normal animal after termination of the fast on the 4th day excreted 7.76, 10.12, 12.66, and 11.45 g. during 4 consecutive 24-hour periods, while the infected animal voided considerably more nitrogen during this time interval, namely, 16.72, 16.10, 15.28, and 17.16 g., respectively. It must be understood, however, that the normal dog actually had fasted for a total of 5 days, even though data for only a 3-day fasting period is shown. The extension of the fast to 5 days was necessary because an infected dog will at times refuse food for as many as 5 days. The results of the analyses after termination of the fast of the normal dog, therefore, were actually those on samples collected after the 5th day of fasting and not after a 3-day fasting period, as might be assumed from the arrangement of the data in Tables I–VII. Repeated trials have shown that a normal dog will return to his normal level of nitrogen, inorganic sulfate, etc., excretion as rapidly after 3 as after 5 days of fasting.

The nitrogen, inorganic sulfate, and organic sulfur excretion increased in all dogs during the infection from 2 to 4 times above normal values, while no significant changes occurred in ethereal sulfate and thiosulfate eliminations (Tables I–V). The values of the latter, although at times appearing slightly larger than the normal ones, vary too much to make them significant. Creatinine excretion remained essentially constant, which therefore served admirably as a check on the completeness of urine collection (Table VII); complete catheterization of dogs with severe infections is, at times, very difficult.

Creatinuria always occurred during the course of the infection. The total amount of creatine excreted in 24 hours during the fasting periods of the illness was at least double that of the normal, not fasting dog, and occasionally 10 times the amount of the fasting dog (Table VI). When

food was finally consumed by the infected dog, a further rise in creatinuria took place. The amount of creatine excreted during the last 24-hour period by the 2 dogs which succumbed to the disease were approximately 20 and 80 times more than the corresponding quantities excreted by the same dogs when normal but fasting. Since excessive creatinuria is considered to be an indication of an increased tissue protein catabolism, a portion of the observed extra urinary nitrogen and inorganic sulfate excreted during infection must have been due to tissue protein breakdown. Somewhat unexpected is the relatively slight rise in extra organic sulfur (Table III) eliminated by the infected animals. In human pneumonia, albuminuria is quite generally observed during the febrile stages of the disease (16, 17) and it was, therefore, expected that a similar observation would be made in these dogs. A number of qualitative tests for proteins were made on the urine of some of the dogs during the acute stages of the disease, while temperatures of above 104°F. prevailed for several consecutive days, but either no positive test or only very weak ones were observed. It must, therefore, be concluded that the extra organic sulfur of dog urine during the acute stages of the disease is not of macro protein nature (negative Heller's ring test; no precipitates with equal volumes of 10 per cent trichloroacetic acid or 20 per cent sulfosalicylic acid added to acidified urine).

Essentially the same results were obtained for all the urine constituents for which analyses were made, whether the dogs received sulfadiazine or were untreated. An especially fortunate case in this respect is Dog 55 where the same dog served as his own control. After an infection of considerable severity from which the dog recovered without drug therapy, the animal was reinfected 6 months later. The reinfection was almost of the same severity as the first one. Sulfadiazine was administered in therapeutic dosage, as shown by blood levels of from 8 to 20 mg.%, beginning with the 25th hour after infection, and the therapy was continued for 5 days (Tables I–VI, Columns 2 and 3 for Dog 55). As is clearly evident, the two infections resemble each other closely in all respects so that the conclusion must be drawn that sulfadiazine does not influence the excretion of extra nitrogen, sulfur compounds, creatine, and creatinine in dogs during Type I pneumococcal pneumonia.

SUMMARY

During Type I lobar pneumonia in dogs, large amounts of extra urinary nitrogen, creatine, inorganic sulfate, and organic sulfur were excreted. No significant changes in creatinine, ethereal sulfate, and

thiosulfate elimination were observed. Sulfadiazine therapy was without influence on the excretion of any of the above constituents, in normal and in infected dogs.

Of six thorough-bred bull terriers used in this investigation, the two male dogs excreted normally about 10 times as much thiosulfate as the females. During fasting periods urinary thiosulfate excretion disappeared completely in all dogs but reappeared within 24 hours after the resumption of food. Sulfadiazine administration had no influence on the level of urinary thiosulfate.

Sulfadiazine administration causes diuresis of considerable magnitude in normal dogs. Food consumption is a necessary condition for this type of diuresis, for if food is withheld from dogs during drug therapy, the amount of urine excreted daily is normal, but becomes abnormally large as soon as food is again consumed.

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Iron Deficiency in Bacterial Metabolism

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Introduction

Our present knowledge of the function of metals in enzyme systems has been derived chiefly by two methods: (1) inactivation of the metal group by specific inhibition; and (2) removal of the metal ion in question from the system, usually by dialysis.

Although the first method may be criticized on the ground that no inhibitor is entirely specific, and many inhibition effects can not be explained adequately, this approach has yielded most of our knowledge of the participation of metals in enzyme action.

The method of removing the metal ion from the system and readdition is probably the best proof that it has an essential rôle, but some metals, especially iron, often require such severe treatment before they become dialyzable that the protein of the enzyme becomes irreversibly denatured.

A third method which has recently been suggested is that of growing the organism under conditions of metal deficiency and comparing its systems with those of the normal cell. This method has the advantage that it may reveal the necessity of a metal ion for the synthesis of a specific enzyme during growth without that metal entering into the structure of the enzyme itself. Owing to the extreme difficulty of obtaining media highly deficient in the trace elements, little work has been done along these lines. The present report concerns iron-deficient metabolism in bacteria grown under conditions of metal deficiency.

Methods

Aerobacter indologenes, Iowa State College 23B, was selected for this study because it grows exceptionally well on the following basal medium under both aerobic and anaerobic conditions:

Dextrose	1.0%
K_2HPO_4	0.4%
KH_2PO_4	0.1%
$(NH_4)_2SO_4$	0.1%
Mg^{++}	10.0 p.p.m.
Zn^{++}	0.01 p.p.m.
Mn^{++}	0.01 p.p.m.
Cu^{++}	0.01 p.p.m.

The traces of iron were removed from this medium by an 8-hydroxyquinoline-chloroform extraction method previously described (19).

The basal medium, without addition of iron produced small but adequate yields of iron-deficient cells of *A. indologenes*.

When it was desired to grow cells containing a normal amount of iron, the above medium was supplemented with fresh solution of ferric chloride to give a final concentration of 0.25 p.p.m. Fe. This is about ten times the minimal normal requirement and less than one-tenth the minimal toxic concentration of iron for *A. indologenes* (20).

Normal aerated cultures incubated at 30° C. for 20 to 24 hours yielded 5–6 g. of cell paste per liter of medium. Iron-deficient aerated cultures required 36 hours' incubation, since the lag phase was 10 to 12 hours longer than that of the normal aerated cultures. The yields varied from 0.5 to 0.7 g. per liter. Cultures were aerated by means of a pyrex capillary tube which introduced a steady stream of fine air bubbles at the bottom of the flask. The air was filtered through a sterile cotton filter tube. This method of aeration did not introduce any iron into the medium.

The media for growth of the normal and iron-deficient anaerobic cultures were cooled rapidly after autoclaving, inoculated, and oxygen-free nitrogen forced through to remove any oxygen. These cultures were incubated 28 and 40 hours and yielded 1.4 to 1.9 and 0.4 to 0.6 g. per liter respectively.

The cells were harvested, washed twice with distilled water by centrifugation and always used within a few hours after harvesting. The normal and deficient cells were treated in exactly the same manner. In the catalase experiments the equivalence of suspensions used was checked by wet weight and turbidimeter. In all other work the dry weights were accurately determined on aliquots of the suspensions used.

Preliminary studies revealed that the extreme cleanliness and care required for production of these iron-deficient cells was not necessary in the operations following harvesting of the cells from the medium. They have no apparent ability to synthesize any of the iron systems studied here excepting during growth.

In previous work (20), it was found that growth of *A. indologenes* was a function of the iron concentration in the medium between 0 and 0.025 p.p.m. The yield of cells obtained in a culture under carefully controlled conditions was found to be a good index of the degree of iron deficiency in the cells. The data are representative of those obtained by examination of a large number of normal and deficient cultures.

Experimental

Catalase Activity. The catalase activity of the normal and deficient cells was measured by the Barcroft-Warburg technic. Cell suspensions contained one gram of cell paste in 5 ml. The cell suspension was placed in the side arm of the vessel and tipped into the substrate after the system had reached equilibrium in the bath. The temperature of the bath in these and all other respirometer experiments was 30.4° C. The catalase activity of the iron-deficient culture was greatly reduced (Table I).

Peroxidase Activity. The peroxidase activity of the cells was measured by their purpurogallin numbers. These values represent the milligrams of purpurogallin formed from pyrogallol by hydrogen peroxide according to the conditions established by Willstätter and Stoll (21). The purpurogallin numbers were determined by use of the chromic acid standard of Bansi and Ucko (2). A standard prepared from purpurogallin obtained by the method of Perkin and Stephen (12)

TABLE I

Catalase Activity of Cells

Time in minutes	Catalase activity, $\mu l.\ O_2$ evolved			
	Normal cells		Fe-deficient cells	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1	86	52	2	3
2	72	46	2	3
3	67	43	2	3
4	65	41	1	2
5	61	39	1	1

Substrate, 1 ml. of 0.6 per cent H_2O_2; cell suspension, 0.25 ml.; phosphate buffer $M/10$ pH 6.8, 0.5 ml.; total volume 2.00 ml.

gave photoelectric colorimeter readings which were 12% lower than those obtained with the chromic acid standard. The latter was considered better for this comparative study. The volume of the reaction solution was reduced to one-tenth (200 ml.) according to present practice, and acetate buffer was substituted for the phosphate. Phosphates usually contain enough heavy metal, especially copper, to give high blanks (1). In each case a portion of the cell suspension tested was boiled ten minutes, cooled, and peroxidase determined immediately; this value was subtracted from that of the viable suspension. Considerable variation in peroxidase activity was found with the different cultures of normal cells. Three separate iron-deficient cultures were examined. Of these, two gave values equal to the boiled controls and the other a value so close to that of the boiled controls that it was hardly determinable by the method used.

It is apparent that the catalase and peroxidase activities of the iron-deficient cells were reduced to about the same extent. Each appears to be less than 5% of the normal values found.

Cytochrome System. There are no data available concerning the type of cytochrome system contained in *Aerobacter*. Observations with suspensions of *A. indologenes* showed that this organism has the two banded spectrum found in *Escherichia coli*. According to Keilin (8) *E. coli* has a primitive form of the cytochrome system which, because it lacks cytochrome c, will not oxidize *p*-phenylenediamine or hydroquinone.

Measurements of the cytochrome oxidase activity of *A. indologenes* cells by oxidation of *p*-phenylenediamine and hydroquinone were negative.

Spectrometric examinations were made on equivalent suspensions of the normal and deficient cells in the reduced state (hydrosulfite added). The normal cells showed a moderate band at 560 mμ and a weak a_1 band at 590 mμ. No absorption bands were visible in the spectrum of the deficient suspensions. The deficient cells were nearly chalk white, whereas normal cells were a dark cream to buff color.

TABLE II

Purpurogallin Numbers of Normal and Fe-deficient A. indologenes Cells

Normal cells	Purpurogallin numbers	Fe-deficient cells	Purpurogallin numbers
1	12.2×10^{-3}	1	0
2	8.1×10^{-3}	2	3.4×10^{-4}
3	11.4×10^{-3}	3	0

Dissimilation. Experiments were carried out on the Barcroft-Warburg respirometer to determine the rates of dissimilation of common substrates by normal and deficient cells. The following conditions were used in this work. Temperature, 30.4° C.; phosphate buffer $M/15$ pH 6.8, 0.5 ml.; substrate $M/10$, 0.5 ml.; 20% cell suspension, 0.25 ml.; final volume 2 ml. In the aerobic experiments alkali with absorbing paper was used in the central well. In the anaerobic work oxygen-free nitrogen (Kendall apparatus) was passed through the respirometer for 10 minutes. Corrections were made for barometric changes and endogenous activity. The results are calculated in terms of Q_{O_2} and $Q_{CO_2+H_2}$ (Tables III to VI).

When the iron-deficient cells were grown aerobically their oxygen uptake with glucose was of the same order as that found with the normal cells. However, when iron-deficient cells were grown anaerobically, their ability to take up oxygen on this substrate was even greater than that of the normal cells grown anaerobically (Table V).

Oxygen uptake with lactate, pyruvate, and acetate was diminished in iron deficiency whether the cells were grown aerobically or anaerobically.

An interesting finding was the complete absence of the anaerobic formate splitting enzyme system in the iron-deficient cells grown under both conditions. The aerobic formate splitting system was also entirely absent in the iron-deficient cells grown aerobically, but was present in a depleted state (20 to 30% of normal) in those grown anaerobically.

TABLE III

Q_{O_2} *Values of Cells Grown under Aeration*

Culture	Glucose	Lactate	Pyruvate	Acetate	Formate
Normal 1	73	38	41	15	60
Normal 2	51	—	53	8	81
Deficient 1	55	11	17	0	1
Deficient 2	68	—	13	—	1

TABLE IV

$Q^{N_2}_{CO_2+H_2}$ *Values of Cells Grown under Aeration*

Culture	Glucose	Lactate	Pyruvate	Acetate	Formate
Normal 1	242	1	126	3	195
Deficient 1	10	1	4	1	1

TABLE V

Q_{O_2} *Values of Cells Grown under Anaerobiosis*

Culture	Glucose	Lactate	Pyruvate	Acetate	Formate
Normal 3	34	27	36	3	36
Normal 4	34	—	36	—	57
Deficient 3	49	15	13	1	10
Deficient 4	56	12	18	1	11

The $Q_{CO_2+H_2}$ values found with all substrates for the iron-deficient cells grown both aerobically and anaerobically were less than 5% of those found with the corresponding normal cells. Q_{H_2} values of the normal cells on formate will be discussed under formic hydrogenlyase activity.

Inhibition by 0.001 *M* KCN showed respiration to be affected in both normal and iron-deficient cells to the same degree (Table VII). Krebs'

TABLE VI

$Q^{N_2}_{CO_2+H_2}$ *Values of Cells Grown under Anaerobiosis*

Culture	Glucose	Lactate	Pyruvate	Acetate	Formate
Normal 3	306	1	108	1	248
Normal 4	434	—	110	—	162
Deficient 3	6	1	3	1	1
Deficient 4	9	—	3	—	2

TABLE VII

*Effect of 0.001 M KCN on Oxygen Uptake**

Culture	Glucose	Lactate	Pyruvate	Acetate	Formate
Normal 1	73	38	41	15	60
Normal 1 + KCN	9	3	6	2	0
Deficient 1	55	11	17	0	1
Deficient 1 + KCN	4	0	3	0	0

* Q_{O_2}.

TABLE VIII

Dehydrogenase Activity (Thunberg Method)

Decoloration time expressed in minutes

Cells	Substrate							
	Glucose	Formate	H_2	Ethanol	Lactate	Malate	Fumarate	Succinate
Normal A	3	6	1	4.5	9.3	30	30	32
Normal B	3.5	6.6	1.2	5.5	11	39	35	38
Deficient A	5.7	116	>180	6.8	10	58	66	98
Deficient B	8	>180	>180	11.2	16	71	74	104
Deficient C	6	120	>180	8.5	17	53	—	—

mixture (9) was used in the wells of those vessels where KCN was present.

Dehydrogenases and Hydrogenase. The dehydrogenase activity of the normal and deficient cells was determined by the Thunberg methyl-

ene blue technic. The cells for these determinations were grown aerobically but without continuous aeration. Each Thunberg tube contained 1 ml. of 1:5000 methylene blue, 1 ml. of phosphate buffer (pH 6.8), 1 ml. of $M/10$ substrate, and 1 ml. of cell suspension containing approximately 30 mg. of cell paste. The tubes were flushed with nitrogen from which the oxygen had been removed by passing it through a Kendall apparatus and a pyrogallol absorption train. In the case of the hydrogenase experiments oxygen-free hydrogen was used as the substrate-atmosphere. The reduction times shown in Table VIII represent 90% reduction of the methylene blue as matched against a 10% control tube without cells.

The glucose, lactic, and ethanol dehydrogenases present in the iron-deficient cells appeared to be practically normal. Succinic and malic dehydrogenases and fumarase appeared somewhat depleted. Formic dehydrogenase was nearly entirely absent, and hydrogenase was hardly detectable. Recently Lipmann (10) has stated that hydrogenase is probably an iron enzyme because it is reversibly inactivated by CO. The absence of this enzyme in iron deficiency further strengthens this view.

Effect of Copper. Elvehjem (5) and Yoshikawa (22) have demonstrated a rôle of copper in the iron metabolism of yeasts. No copper effect in *A. indologenes* was obtained.

The 8-hydroxyquinoline extraction method for removal of iron from the basal medium also removes copper. When the medium was purified in the customary manner, it was found to be copper-deficient for the mold *Aspergillus niger* which requires 0.04 p.p.m. (14), but not for *A. indologenes*.

When *A. indologenes* was grown on the purified basal medium to which had been added adequate iron but no copper, its metabolism was apparently the same as when it was grown in the presence of both iron and copper (0.01 p.p.m. Cu). Nevertheless, it is not at all improbable that copper has a rôle in the metabolism of this organism. The iron requirement of *A. indologenes* is very low (0.025 p.p.m.). If its copper requirement is in the ratio of 1:10 with its iron requirement as in the case of the yeasts that have been studied, it should probably need only 0.0025 p.p.m. Cu. On this basis a medium would have to contain less than 0.001 p.p.m. of Cu to show its deficiency in *A. indologenes*. This condition would be extremely difficult to obtain.

Fermentation Analysis. Preliminary analysis of the anaerobic fermen-

tation of glucose by iron-deficient *A. indologenes* yields the following results as:

Millimoles of Product per 100 Millimoles of Glucose Fermented:

CO_2	H_2	Formic	Acetic	Alcohol	Lactic	Succinic	2,3-Butylene glycol
13.6	0	92.0	33.6	37.6	88.3	0	5.7

These conform with other findings in this investigation.

Discussion

Several interesting generalizations may be made concerning iron deficiency in the simple heterotrophic bacterial cell.

(1) In the iron-deficient bacterial cell certain iron-containing enzymes may be depleted or entirely absent without affecting the activity of other enzymes present. Iron deficiency apparently does not produce a general feebleness of the bacterial cell. At least this does not occur in the facultative organisms of the coli-aerogenes group.

(2) There appears to be a selective distribution of the iron in the iron-deficient cell. The iron needs of the cytochrome system appear to be met first. Other apparently less vital enzymes including catalase and the peroxidatic hemins may show less than 5% of normal activity in an iron-deficient cell having an almost normal cytochrome system. Under the same conditions other secondary enzyme systems involving iron may be depleted or entirely absent. The formic acid splitting enzymes are of this type.

The low content of peroxide-activating enzymes in the iron-deficient cell may indicate that they are normally present in excess, or that they are less vital to the cell than has been believed.

The absence of cytochrome absorption bands in the iron-deficient cells is hard to reconcile with the normal oxygen uptake of these cells on glucose, and the amount of cyanide sensitive respiration found. The normal cell may contain more of these pigments than it uses during respiration on glucose. *A. indologenes* has a weak cytochrome spectrum; other hemins closely associated with cytochrome may normally contribute to these absorption bands at 560 mμ and 590 mμ. In the absence of the hemin bands the weak cytochrome bands may not be of sufficient intensity to be visible. There is also the possibility that a cyanide-sensitive respiration other than that of cytochrome may be present in iron deficiency.

The increased Q_{O_2}-glucose values found with iron-deficient cells grown anaerobically suggest existence of a mechanism involving iron which normally inhibits oxygen uptake in the anaerobically formed cell. This mechanism may be an iron-containing Pasteur enzyme similar to that described by Stern and Melnick (17).

The lack of CO_2 liberation by the iron-deficient cells is probably due to the absence of the formate-splitting enzymes. If a small amount of CO_2 is formed by decarboxylation, it would probably be utilized by the organism.

Formic Acid Enzymes. The absence of formic acid splitting enzymes in iron deficiency may be interpreted in at least four ways.

(a) Iron may be a functional component of these enzymes.

(b) Iron may be a non-functional but structural component of the enzyme molecules, in the sense of Green (6).

(c) Iron may be necessary in the synthesis of these enzymes during growth, without entering the synthesized molecules.

(d) Iron may be a component of a system associated with the normal functioning of the formic enzymes. Catalase, for instance, may be concerned.

Further work is necessary to establish the actual rôle of iron in these enzymes but there seems to be no doubt that the metal is essential. It has been suggested that copper is the metal in formic dehydrogenase (4). No evidence of this could be found with *A. indologenes.*

The necessity for iron in the system responsible for formation of hydrogen gas from formate is very noticeable. On the basis of the present study and others, it is possible to propose a tentative hypothesis concerning the nature of this system.

Quastel and Whetham (13) discovered an enzyme in *E. coli*, which they called formic dehydrogenase, capable of catalyzing the reaction:

$$HCOOH + A \rightarrow AH_2 + CO_2$$

where A represents a hydrogen acceptor.

Investigation of hydrogen gas formation led Stephenson and Stickland (18, 15) to the discovery of another enzyme, hydrogenase, which is capable of activating molecular hydrogen according to the following reaction:

$$H_2 \xrightarrow{-2e} 2H^+$$

These investigators postulated that molecular hydrogen was liberated from formate by the combined action of the two enzymes according to the reactions

$$HCOOH \rightarrow 2H + CO_2$$
$$2H^+ \xrightarrow{+2e} H_2$$

They stated that if an organism could be found which liberated hydrogen gas from formate without possessing both formic dehydrogenase and hydrogenase the above scheme would be untenable.

Stephenson and Stickland later claimed to have found that *Bacterium lactis aerogenes* (*Aerobacter aerogenes*) produced hydrogen gas without apparently possessing the enzyme hydrogenase. They also found that *Shigella dispar* possessed both hydrogenase and formic dehydrogenase but was not able to liberate gas from formate. They therefore postulated a new enzyme (16) capable of catalyzing the reaction

$$HCOOH \rightarrow H_2 + CO_2$$

This hydrogen liberating enzyme they named formic hydrogenlyase, meaning literally "to loosen hydrogen."

The problem was recently taken up by Ordal and Halvorson (11) who have shown that *A. aerogenes* does possess the enzyme hydrogenase. There is therefore no known organism lacking these enzymes, which is able to produce hydrogen from formate. These authors also found one strain of anaerogenic *E. coli* which possessed both formic dehydrogenase and hydrogenase.

Stephenson and Stickland found that *E. coli* grown in plain broth was not able to liberate hydrogen, but when grown in formate or carbohydrate broth, it developed this latent power. Ordal and Halvorson have suggested in view of this fact and their findings, that there may be a third factor concerned. When the cells are grown on plain broth this factor is not synthesized although the other two enzymes concerned are found to be present.

The findings reviewed above, together with data found in iron deficiency and certain inhibitor experiments make possible the following hypothesis.

(1) Production of molecular hydrogen from formate by certain bacteria is accomplished by the combined action of (a) formic dehydrogenase; (b) an intermediate electron mediator; and (c) hydrogenase

according to the reactions

$$HCOO^- + H_2O \rightarrow HCO_3^- + 2H^+ + 2e$$
$$2H^+ + 2e \rightarrow H_2$$

(2) The electron carrier in question contains functional iron, probably in a manner similar to cytochrome.

Evidence for the above hypothesis is as follows.

(1) All bacteria which produce molecular hydrogen from formate appear to possess both formic dehydrogenase and hydrogenase.

(2) Numerous cases are known where *E. coli* possesses formic dehydrogenase and hydrogenase when grown in plain broth but lacks a factor necessary for production of gas from formate. This factor, however, appears when these organisms are grown in formate or carbohydrate broth.

(3) Investigations with inhibitors have shown that the formic hydrogenlyase system is a complex one. It is sensitive to poisons of all kinds (16). It is affected not only by typical dehydrogenase inhibitors (narcotics) but also by common inhibitors of metal catalysis, *e.g.*, KCN and CO. This system is extremely sensitive to cyanide, being inhibited 50% by a concentration of 10^{-5} *M* and 100% by 10^{-3} *M*. CO is known to act on hydrogenase, but neither this enzyme nor formic dehydrogenase is affected by cyanide excepting in very high concentration (13, 15).

(4) *A. indologenes*, when grown on a basal medium deficient in iron but with adequate amounts of other well known enzyme-forming metals, copper, zinc, manganese, and magnesium, is unable to produce hydrogen from formate. The same strain grown under the same conditions and on an identical medium but to which has been added only iron, forms hydrogen in normal quantities.

(5) Small, but significant hydrogenase and formic dehydrogenase activities are found in cells which are not sufficiently iron-deficient to bring about complete suppression of these enzymes, but these same cells do not form hydrogen from formate. No hydrogenlyase activity (less than 1%) has ever been found in any of the iron-deficient cultures examined. This system is more easily suppressed by iron deficiency than any other system studied.

(6) It has been claimed that formic hydrogenlyase activity does not occur in *Aerobacter* cells grown under conditions of continuous aeration (15). With *A. indologenes* it was found that this system is formed during

vigorous aeration (alundum ball aerator) when adequate iron has been added to the medium. Separate experiments were carried out to determine the degree of its formation during aeration. Four liters of the basal medium to which had been added 0.5 p.p.m. iron and 0.1% sodium formate were divided into two equal parts. One portion, after autoclaving, was cooled rapidly, inoculated and made anaerobic by passing oxygen-free nitrogen through it. The other portion was inoculated and kept aerobic during growth by continuous vigorous aeration through a porous alundum ball. The cells from these cultures were tested on formate for hydrogen formation (alkali in central well). The Q_{H_2} values found with the cells grown anaerobically were 119–128; those found with the cells grown aerobically were 118–124. Lack of the formic hydrogenlyase system in *Aerobacter* cells from aerated cultures as reported by Stephenson and Stickland (15) may have resulted from a deficiency of available iron for its formation. During aerobic growth the cytochrome system, catalase, peroxidase, and probably other iron hemins will be formed in maximal amounts, thus more iron is required than when conditions of growth are less aerobic. If adequate available iron is not present under these conditions, the hydrogen forming system may not be synthesized, as in the case of the iron-deficient cells studied. This phase of the problem is under further investigation.

(7) Borsook (3) has found evidence that an electron carrier operates between the system formate ↔ bicarbonate and pyruvate ↔ lactate. Toluene treatment was found to destroy this carrier, but the system could be restored by addition of the dye methylene violet or janus green ($E_0' = -0.26$ v). It has been found that toluene also inactivates the formic hydrogenlyase system (7). That toluene acts on the proposed carrier is likely since it has no effect on hydrogenase or formic dehydrogenase.

A final proof of the existence of the proposed carrier might be obtained by its separation from hydrogenase and formic dehydrogenase.

Summary

A study of the effect of iron deficiency on the enzyme systems of *A. indologenes* has shown that catalase, peroxidase, formic hydrogenlyase, formic dehydrogenase, and hydrogenase activities were suppressed by iron deficiency. The cytochrome bands were not visible in the iron-deficient cells. Analysis of the fermentation of glucose by the iron-deficient cells showed high yields of formic and lactic acids, low CO_2

production and no succinic acid or hydrogen gas. It is believed that an iron-containing electron mediator operates in the formic hydrogenlyase enzyme system.

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The Fatty Materials in Diapausing Codling Moth Larvae (Carpocapsa Pomonella L.)*

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Introduction

The codling moth, *Carpocapsa pomonella* (L.) still continues to be the primary insect enemy of apples throughout the apple growing regions of the United States and Canada. Many detailed studies of the life history have shown that the number of generations in any given season is variable, being dependent upon the prevailing climatic conditions. It has also been revealed that a certain number of the larvae from each generation enter a diapause or resting stage in which they remain until the following spring, at which time they pupate and emerge as adults. As a general rule all, or nearly all, of the last generation enter this stage. It has been pointed out, also, that a certain number of these diapausing individuals may not pupate in the spring following their entrance into this stage, but may carry over for another year.

The phenomenon of diapause is generally thought of as a biological adaptation to possible adverse conditions. These conditions cannot be considered as the only motivating factors as they cannot explain why all individuals in a given generation do not react the same and enter either one state or the other. Attempts to induce artificially a diapause state among insects have been quite numerous, especially with the sugar beet webworm, *Loxostege sticticalis* (L.) whose life history is similar in general detail to that of the codling moth. Such work with the webworm has been based largely on the assumption that some ecological factor or combination of factors is responsible for the condition. These investigations have not produced any very conclusive results as yet and still leave much to be learned regarding the phenomenon of diapause.

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Pepper (1937) was able to demonstrate that the diapause stage in the sugar beet webworm could be readily broken when the individuals were subjected to low concentration of the vapors of certain chemicals. These chemicals all proved to be fat solvents. Those not possessing this property produced no effect.

Together with many other observations, the fact that the diapause condition can be prematurely broken in the sugar beet webworm prepupa by subjecting it to vapors of various fat solvents lead Pepper and Hastings (1943) to suggest that some connection, either direct or indirect, may exist between diapause and the activities of the lipid materials in the fat bodies. Preliminary studies indicated that the codling moth did not respond to these diapause-breaking substances as did the webworm. Since the life histories of these two insects are similar, it was thought that a comparison of the fatty materials present in diapausing individuals of both insects might give some information which could account for the differences in their behavior.

Experimental

Codling moth larvae* were collected by banding apple trees with strips of corrugated paper, the bands being set out at such a time that the larvae from the autumn generation could enter the folds of the paper and construct the silken cases in which they normally overwinter.

Attempts to Break Diapause

These larvae (now prepupae) were kept at room temperature to eliminate severe temperature changes which are known to affect the resting period. These prepupae were used soon after collection to study the effect of a series of chemical vapors on the breaking of the diapause state. They were treated in fumigation flasks with varying sublethal concentrations of vapors for different lengths of time and were then allowed to spin up in thin, specially constructed, glass covered cells where their behavior could be observed. The cells were kept in a constant temperature cabinet at 28°C. The list of chemicals used included those employed by Pepper (1937) on webworm prepupae: xylol, carbon tetrachloride, diethyl ether, ethylene dichloride, ethyl bromide, and ethyl iodide: those which have been found to be highly effective against plants;

* A large number of the codling moth larvae were obtained through the kindness of E. J. Newcomer of the Bureau of Entomology and Plant Quarantine, Yakima, Washington.

chloroform, ethyl disulfide, propylene chloride, acetaldehyde, furan, s-tetrachlorethane, dichlorethylether, and trichlorethylene: and one which the authors were informed had been successful in breaking the codling moth rest period; tergitol, a trade name. This latter material is a non-volatile oily substance and was used by allowing various amounts to be absorbed on the walls of holding cells. None of these chemicals proved to have any effect in prematurely breaking the diapause condition in codling moth larvae.

General Analyses

Prepupae, collected as outlined above, were stored at 2°C. until they were used in making the following analyses:

The protein nitrogen was determined on fat-free individuals by an adaptation of the simplified macro-Kjeldahl method of Folin and Wright (1919). The factor 6.25 was used in calculating the crude protein content of the material. Moisture was determined by desiccating the larvae *in vacuo* at 40°C. to constant weight. They were found to contain 54 per cent free moisture. When calculated on a dry weight basis they contained 44.2 per cent of ether extractable fats and 39.9 per cent protein, the remaining 15.9 per cent being chitin, ash, and other non-protein or fat-like substances.

*Fat Analyses**

The major portions of the following analyses were made on individuals which had not been in the diapause stage for a period of over six months. For comparative purposes, however, a limited number of determinations were made on fat obtained from some larvae which had been held in this resting stage for a period of three years.

Before the fats were extracted the larvae were opened and dried to less than one per cent moisture content in a vacuum desiccator over sulfuric acid at 25°C. The fats were then extracted in a Soxhlet extractor using diethyl ether. The ether was then removed *in vacuo* in a stream of nitrogen, taking care not to let the temperature rise above 35°C., and the residue redissolved in aldehyde free anhydrous diethyl ether. The container was then flushed out with nitrogen, tightly stoppered and the fat stored at 2°C. until the analysis could be completed. This treatment held both oxidation and hydrolysis of the fats to a minimum as judged by frequent determinations of the iodine number throughout the course of the study.

Analysis of the Total Fats

Iodine numbers were determined by the Wijs (1929) method, and a modification of the Kaufmann (1926) method was used for determining the thyocyanogen num-

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* Methods of analysis of fats outlined under this heading are given in detail by Pepper and Hastings (1943).

bers. The acid values were determined in accordance with the directions given in Jamieson's Vegetable Fats and Oils (1932), while the saponification or Koettstorfer number was determined by the method given in Methods of Analysis A.O.A.C. (1940).

The ester value was determined by subtracting the number of milligrams of KOH required to neutralize the free fatty acids, in one gram of sample, from the saponification value.

The unsaponifiable matter was determined according to the method of Allen and Thompson as described by Lewkowitsch (1909).

Assuming that oleic and linoleic acids were the ones present in the larval fat the percentage composition of the glycerides of oleic and linoleic and saturated acids was calculated by the formula given in Methods of Analysis A.O.A.C. (1940).

The results of these analyses on the total fats and the calculations are shown in Table I.

TABLE I

Results of Analysis on the Total Ether Extractable Fatty Materials Obtained from Diapausing Larvae of the Codling Moth

Iodine number	100.0
Thiocyanogen number	82.0
Saponification number	202.0
Acid value	8.1
Ester value	193.9
	per cent
Unsaponifiable matter	1.34
Percentage of linoleic acid glycerides	20.8
Percentage of oleic acid glycerides	74.3
Percentage of saturated acid glycerides	3.6

Analysis of the Total Fatty Acids

A modification of the Twitchell (1921) method was used in separating the saturated from the unsaturated fatty acids in which the lead salts of the saturated fatty acids were precipited and filtered off.

The unsaturated fatty acids were obtained from the filtrate in the previous separation, the acids being liberated by the addition of dilute HNO_3.

On the assumption that only monobasic acids were present, the mean molecular weight of the total fatty acids was calculated from the values obtained in the acid value determinations, by substitution in the formula

$$\text{C.C. KOH (from acid value)} \times \text{Normality} = \frac{\text{gms. of material}}{\text{equivalent weight}} \times 100$$

The results of the analyses on the total fatty acids and the calculations are shown in Table II.

Analysis of the Saturated Fatty Acids

The results of these determinations are shown in Table III.

TABLE II

Results of Analysis of the Total Fatty Acids Obtained from Diapausing Larvae of the Codling Moth

Iodine number	105.0
Thiocyanogen number	87.0
Acid value	205.0
Mean molecular weight	273.1
	per cent
Percentage of unsaturated fatty acids	88.0
Percentage of saturated fatty acids	12.0

TABLE III

Results of Analysis of Saturated Fatty Acids Obtained from Diapausing Larvae of the Codling Moth

Iodine number	2.6
Acid value	234.0
Mean molecular weight	210.7

TABLE IV

Results of Analysis of Unsaturated Fatty Acids Obtained from Diapausing Larvae of the Codling Moth

Iodine number	116.5
Acid value	204.3
Mean molecular weight	240.3
	per cent
Percentage of linolenic acid	8.4
Percentage of linoleic acid	11.4
Percentage of oleic acid	68.2

Analysis of the Unsaturated Fatty Acids

Assuming that the unsaturated fatty acids consisted of a mixture of oleic, linoleic, and linolenic acids as was found to be the case by Pepper and Hastings (1943) for the webworm prepupae, the proportions of these acids present in the mixture were calculated by the method of Kaufmann and Keller (1929) using the equations given in Methods of Analysis A.O.A.C. (1940).

The results of the analysis and calculations on the unsaturated fatty acids are shown in Table IV.

Discussion

A comparison of the above data on diapausing codling moth larvae with those obtained by Pepper and Hastings (1943) for the comparable stage, the prepupa, of the sugar beet webworm can be obtained from Table V. This indicates a considerable difference in their percentage composition of moisture, ether extractable fats and crude protein. In both cases the fat and protein were calculated on a dry weight basis. The fat reserve in the codling moth prepupae is over 20 per cent greater than in the webworm prepupae. Over 98 per cent of the moisture free materials in the webworm prepupae consisted of protein and fat while only 84 per cent of the dry ingredients in the codling moth larvae can be accounted for by these materials. Chitin and ash could only account for from one to two percent of the remaining 15.9 per cent of unidentified materials in the codling moth. Since this rather marked difference in the general composition of the comparable stages of the two insects cannot be identified as ether extractable fats or protein these materials are probably carbohydrate in nature although such things as lipo-proteins, phospholipids, sterols, etc., would not be accounted for in their entirety in the ether extractable portion.

TABLE V

Comparative Analyses of the Codling Moth and the Sugar Beet Webworm

	General Analyses			Total Fats		Total Fatty Acids			
	Moisture	Ether Extractable Fats	Crude Protein	Acid Value	Saponification Value	Unsaturated Fatty Acids	Oleic	Linoleic	Linolenic
	per cent	*per cent*	*per cent*			*per cent*	*per cent*	*per cent*	*per cent*
Codling Moth...	54.0	44.2	39.9	8.1	202.0	88.0	68.2	11.4	8.4
Sugar Beet Webworm*....	37.6	12.1	86.7	23.8	187.1	75.0	38.3	13.3	23.4

* Data from Pepper and Hastings (1943).

A comparison of the nature of the total fats showed that the webworm prepupae contained more free fatty acids than the codling moth prepupae, as indicated by their acid values, a fact which is further brought out by their saponification values. The sugar beet webworm prepupae con-

tained a smaller percentage of unsaturated acids, a large proportion of these acids, however, belonged to the more highly unsaturated acid series than was the case with the codling moth.

Iodine numbers on the fats from codling moth larvae which had remained in the diapause stage for three years dropped from 100.0 to 84.0, indicating that the unsaturated acids were the ones being utilized to the greatest extent during this period. The amount of free fatty acids, however, remained constant, no difference being observed in their acid values.

According to previous work involving detailed studies on the changes in fatty materials throughout the life cycle of the webworm (Pepper and Hastings, 1943) there is a definite change in the percentage composition of the total fatty acids during the overwintering period of the prepupae and before the transformation to the pupal stage. During the five months which these overwintering webworm prepupae were in this stage, the iodine number of their total fatty acids increased from 122.6 to 125.7. Further analysis showed this to be due to the utilization of linoleic acid resulting in a higher percentage, in the remaining fatty acids, of the more highly unsaturated linolenic acid. It is possible that if these webworm prepupae had been held in this state for a period of time comparable to the three years that the codling moth prepupae were held, the more unsaturated acids would have been utilized and the iodine number lowered.

The above comparison of the data on the two species of insects does not reveal any differences in the composition of their fats which might account for the observed differences in the behavior of their diapausing forms to the action of various fat solvents. The general analysis, however, showed that the composition of the two insects differed considerably, 98.8 per cent of the dry matter in the webworm prepupae being accounted for as crude fat and protein whereas only 84.1 per cent of the diapausing codling moth consisted of these materials. A knowledge of the composition of the unidentified materials may reveal information which would be helpful in explaining, at least in part, the reasons why chemicals which will readily break the diapause in the webworm prepupae have no effect on the comparable stage in the codling moth.

Summary

A general analysis of the codling moth larvae (*Carpocapsa pomonella*), when in the diapause stage, showed them to contain 54.0 per cent mois-

ture. When calculated on the dry weight basis they contained 44.2 per cent crude fats and 39.9 per cent crude proteins.

The following constants were determined on the crude fats: Iodine number, thiocyanogen number, saponification number, acid value and ester value. The percentage unsaponifiable matter was also determined. From the above data the percentage glycerides of oleic, linoleic, and the saturated fatty acids was calculated.

The above constants were then determined on the total fatty acids, and their mean molecular weight calculated.

The saturated and unsaturated fatty acids were separated and their iodine numbers and acid values determined. From these data their mean molecular weights were calculated. The percentage of oleic, linoleic, and linolenic acids in the unsaturated fatty acid fraction were also calculated.

A comparison of these data is made with those obtained from the comparable state of development in the sugar beet webworm (*Loxostege sticticalis*).

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A Differential Analysis of Mixtures of α- and β-Estradiol in Urine Extracts*

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The relative proportions of α- and β-estradiol present in human pregnancy urine and in the urine of rabbits following estrone administration may be estimated by a method (1) which requires the determination of the change in estrogenic activity of the estradiol fraction (non-ketonic, weakly acidic phenols) following oxidation.

Experimental

Collection, Hydrolysis, and Extraction of Urine

A. Human pregnancy urine.[1] The urine was collected during the fifth to seventh months of pregnancy; toluene was used as a preservative. Hydrolysis was carried out within three days after collection by boiling with 15 per cent concentrated HCl (by volume) for 7 minutes. The hydrolyzate was rapidly cooled and extracted with ethyl ether.

B. Rabbit Urine. The urine of two adult female rabbits which had been injected with 36 mg. of estrone acetate dissolved in olive oil was collected for four days and seven hours. The urine was stored in the refrigerator until the collection was completed. Hydrolysis was then performed by adjusting the pH of the urine to 1.6–1.8 with concentrated HCl and autoclaving for 30 minutes at 15 pounds pressure. Ethyl ether was the extractive employed.

Estradiol Fraction

Essentially the same procedure was followed in working up both types of urine. The phenols were obtained as usual, i.e., by alkali extraction from ether solution. Fractionation into ketones and non-ketones was effected with the aid of Girard's reagent T (2). Distribution of the non-ketones between benzene and 0.3 *M* Na_2CO_3 gave the "estradiol" and "estriol" fractions respectively (1). The estro-

* Aided by grants from G. D. Searle Company and the Committee for Research in Problems of Sex of the National Research Council.

[1] The specimens were kindly supplied by Dr. Paul P. Montag; 92 liters were collected.

genic activity of the estradiol fraction was 11,500 R.U. for the human pregnancy urine and 240 R.U. for the rabbit urine; bioassay was performed on spayed adult rats by a method (3) employing the vaginal smear technique. In a control experiment it was found that these rabbits excreted approximately 7.6 R.U. daily; oxidation of the estradiol fraction gave similarly low values.

Analysis of the Estradiol Fraction

Aliquot portions of this fraction were oxidized and the change in estrogenic activity determined. The results are given in the accompanying table. The theory underlying the analysis as well as the chemical procedures involved has been previously described (1).

Estriol Fraction (non-ketonic, strongly acidic phenols) of Rabbit Urine

This fraction of the post-injection urine assayed for 3,260 R.U. The estrogenic activity partitioned between equal volumes of benzene and 0.3 *M* Na_2CO_3 in the ratio of 3.2%:96.8%. Mather (4) found that 2.5% of the activity remains in the organic phase if pure estriol is used.

Discussion

The isolation of α-estradiol from human pregnancy urine has been previously reported (5); the presence of β-estradiol was not contraindicated. The data in this report indicate that β-estradiol is present in very small proportion, if at all, in this source. The conversion factor for the estradiol fraction (see Table) closely approaches that for pure α-estradiol; a considerable degree of differentiation results from the fact that the conversion factor for pure β-estradiol is over a hundred-fold that for pure α-estradiol (1).

The β-isomer of estradiol has been isolated from the urine of rabbits following estrone administration (6), (7). If α-estradiol is injected, the β-isomer is isolated in preponderant amount from the urine (7), (8). The data in this report indicate that very little or no α-estradiol is present in the urine of rabbits following estrone injection. The conversion factor for the estradiol fraction in this instance (see Table) closely approximates that for pure β-estradiol.

The need of a method for the differential analysis of mixtures of the estradiol isomers is apparent if certain estrogen metabolism studies are to be pursued, e.g. in perfusion experiments where the quantity of estrogen is inadequate for isolation. The results described in this report may therefore be regarded as a demonstration of the applicability of the analytical method employed. The method serves also to identify the non-ketonic estrogens under examination; it may be compared, in a

converse sense, to the method used by Westerfeld, *et al.* (10) for the identification of ketonic estrogens wherein the change in activity following reduction of the estrogen was determined. Another method for the analysis of mixtures of α- and β-estradiol has been briefly described by Doisy, Thayer, and Van Bruggen (9). In it a differential mouse smear assay procedure is followed; the total estrogenic potency is determined

TABLE I

Analysis of Urinary Estradiol Fractions

(Aliquot portions were oxidized.)

Source of fraction	Estrogenic Activity			Conversion factor*
	Before oxidation	After oxidation		
		non-ketones	ketones	
	Rat units	*Rat units*	*Rat units*	
Human pregnancy urine	2300	1200	111	0.100
	2300	1040	100	0.079
Average.				0.090
Urine of rabbits injected with estrone	96	45	800	15.7
	96	47	962	19.7
Average†.				17.7
Crystalline α-Estradiol (1)				0.11
Crystalline β-Estradiol (1)				16

* Conversion Factor =

$$\frac{\text{ketonic rat units (after oxidation)}}{\text{rat units (before oxidation) minus non-ketonic (after oxidation) rat units}}$$

† The oxidized fraction was treated twice with the ketone reagent.

by two methods—one using aqueous solutions for injection, the other solutions in oil.

The conversion of estrone into estriol in the female rabbit was first postulated by Pincus and Zahl (11) but attempts at the isolation of estriol from the urine after the administration of estrone (6), (7) or of α-estradiol (7), (8) have been unsuccessful. It appears, nevertheless, from the present study that female rabbits, injected with estrone, excrete in the urine an estrogen with physical and chemical properties resembling those of estriol.

Summary

1. The application of a method for the differential analysis of mixtures of α- and β-estradiol in urine extracts is described. The results indicate that:

a. The estradiol fraction of human pregnancy urine consists exclusively, or almost so, of the α-isomer. (The non-ketonic, weakly acidic phenolic estrogens are referred to as the estradiol fraction).

b. The estradiol fraction of the urine of rabbits injected with estrone consists exclusively, or almost so, of the β-isomer.

2. Female rabbits injected with estrone excrete in the urine an estrogen with physical and chemical properties resembling those of estriol.

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Convenient Method for Deproteinization

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Introduction

The elimination of proteins from liquids of physiological origin can be carried out in neutral, acid, or alkaline media. For preparative and analytical purposes the deproteinization in acid solution has been adopted because the so called acid-soluble fractions of the metabolites have attained increasing importance.

A great number of acids, especially organic acids, have been tested as protein-precipitating reagents.

Trichloroacetic acid, first introduced by Obermayer (1), has been the most widely used up to the present time and is considered indispensable, especially for the examination and isolation of acid-soluble metabolism products (phosphorylated carbohydrates, their transformation products, coenzymes, *N*-phosphoryl-derivatives, etc.).

However, the use of trichloroacetic acid is accompanied by certain disadvantages. It is known that when this acid is used the copper methods indicating the presence of reducing substances are made ineffective, the formation of cuprous oxide being hindered (2). Tryptophan tests with bromine or chlorine water (Tiedemann and Gmelin, 1826) and with formaldehyde (Voisenet) cannot be made. The Millon reaction is positive in the beginning, but subsequently the cherry-red color disappears. The stability of the carbohydrate phosphates against $CCl_3 \cdot COOH$ is not as definite (3) as it is generally assumed to be. The trichloroacetic acid cannot be removed from solutions by precipitation.

It may be noted that trichloroacetic acid not only affects some indicators, but also interferes with the Fehling and other copper methods as well as with the two mercury tests mentioned below. Both the alkaline mercuric cyanide and the mercuric iodide solutions, when heated in the presence of trichloroacetate *per se*,

form white precipitates with the evolution of gas (CO, CO_2). It is also difficult to remove by evaporation.

Using the old observation of Dumas (1839) that trichloroacetic acid is decomposed on boiling into chloroform and carbon dioxide, Hiller and Van Slyke (6) have proposed the elimination of trichloroacetic acid from the filtrates by boiling for 15 minutes. In this way, only 70% of the titrable acid is removed. HCl is formed, too, as shown by Lossen (7) and also CO and CO_2, as confirmed by Beckurts and Robert (8). If these facts are disregarded, the boiling method cannot be used when such sensitive materials as phosphorylated carbohydrates are to be isolated. Such substances are destroyed by boiling acids.

We wish to state that 2,2,3-trichlorobutyric acid, $CH_3 \cdot CHCl \cdot CCl_2 \cdot COOH$, interferes with these reactions also. Trichlorobutyrate itself reduces mercury salts. It interferes with the bismuth reaction in the same way as does trichloroacetic acid. The acid interferes with the action of alkaline copper solutions with the evolution of gas and abolishing the precipitation of Cu_2O. We are indebted to the Pennsylvania Salt Manufacturing Company, Philadelphia, for a sample of this acid.

Deproteinization with perchloric acid ($HClO_4$) shows itself to be free from these disadvantages. Perchloric acid, which apparently has not previously been used for this purpose, precipitates the common proteins. It interferes neither with the copper reduction tests for sugars nor the analytical determination of carbohydrates by means of the Knapp, the Sachsse, or the Boettcher-Almén-Nylander reagent. Perchloric acid does not affect either the Millon test or the halogen-water reaction on tryptophan if the high acidity is first eliminated by addition of an approximately equivalent amount of sodium acetate. The halogen tests are most sensitive in acetic acid solution, especially if the chromogen is extracted with ethylacetate subsequently (4).

Although perchloric acid, in general, does not have oxidative properties, occasionally it might be desirable to remove it from the solution. This can be done by neutralization with potassium hydroxide, carbonate, or acetate and the addition of alcohol, the potassium perchlorate thus being quantitatively precipitated. All other salts of perchloric acid, except the rubidium and cesium salts, are readily soluble in water, and nearly all of them in alcohol and in acetone. This fact has already proved to be of use in the preparation* of pure calcium or barium salts of phosphorylated sugars (5).

* For these and similar purposes metaphosphoric acid, which has been known, since its discovery by Berzelius and Engelhardt, 1816, as one of the best protein-precipitating reagents, cannot be used; besides many of its salts are insoluble in water, but all of them are in alcohol. Furthermore, the solutions of metaphos-

It should be added that, in contrast to trichloroacetic acid U. S. P. which sometimes contains sulfuric acid, perchloric acid of high purity is on sale and at a much lower price.

No investigations have been made concerning the problem as to whether the perchloric acid forms insoluble protein salts or merely produces coagulation.

Experimental

A 1 or 2 *M* perchloric acid solution (about 10 or 20%) may be employed. A solution of trichloroacetic acid of the same molarity was used as a comparison. Generally the biuret reaction (or the Millon test) was employed to indicate the presence or absence of protein material in the filtrates.

The precipitation can be effected at room temperature. Filtration or centrifugation should be done, if after short shaking the floccules conglomerate. If very sensitive substances are to be isolated from the protein free filtrate, the whole procedure should be effected at low temperature.

The following minimum effective concentrations of perchloric acid were obtained. They are expressed as the minimum per cent by weight of acid in the protein-acid mixture which will give negative filtrates by the biuret test.

Protein solution	Minimum effective concentration
	per cent
Ovalbumin (1–5%)	1.1
Caseinate (2–5%)	6.7
Casein-free milk filtrate (diluted 1:1)	3.3
Blood (beef, defibrinated) (diluted 1:10)	1.6
Yeast extracts (diluted 1:1)	6.7

The yeast extracts were prepared by the commonly used method for the preparation of zymase solutions, according to Ivanov and Lebedev. Upon precipitation with perchloric acid, the yeast extracts yielded filtrates with which it was impossible to employ the biuret or Millon test, owing to the presence of peptone-like split-products which are not precipitated by acids. The effectiveness of perchloric acid is shown by testing the filtrate with trichloroacetic acid for further precipitation, if there is any. This test was negative. The yeast extracts showed ex-

phoric acid are unstable. In certain cases, hydrobromic or fluoboric acid may be employed for precipitation of proteins.

actly the same results of complete precipitation of proteins with both acids.

As mentioned above, the acid extracts of cell material are used extensively to isolate and differentiate certain metabolites such as phosphorylated products. We have ascertained the applicability of perchloric acid for these purposes with complete success. We prepared for instance extracts from fresh yeast cells with perchloric acid. In the filtrates we could demonstrate the presence of carbohydrate phosphates and other phosphorus-containing substances which are not extracted by water. Since the salts of perchloric acid are soluble in water and in ethanol, the salts of alkaline earths of the above phosphorus-esters can be isolated from the filtrates in a simple way.

We obtained incomplete or no precipitation of various peptones. Similar results were obtained with albumoses.

Ovomucoid present in natural egg white as well as in commercial preparations of ovalbumin is not precipitable either by perchloric acid or by trichloroacetic acid. This protein is precipitable only by alcohol in strong concentrations, phosphotungstic acid, and tannic acid (9, 10). It is possible to separate the precipitable ovalbumin from the ovomucoid by the use of perchloric acid.

Summary

Perchloric acid is remarkably effective as a precipitant for proteins. This reagent is a pure and cheap commercial product. Perchloric acid performs the same functions as trichloroacetic acid, at the same time being free of the disadvantages of the latter. It is perfectly suited for extraction and isolation of the acid-soluble metabolites.

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The Relation of Thiamine to Blood Regeneration

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In a recent paper (1) we demonstrated in dogs a relation between the level of riboflavin intake and the rate of blood regeneration. A similar technique has been used for thiamine but no direct effect of this vitamin on blood regeneration has been obtained. The experimental results are recorded very briefly in this paper.

Experimental

Eleven mongrel dogs were used. Five adult dogs (Nos. 3, 6, 190, 191, and 202) had previously been used in another experiment and were allowed four weeks on the complete basal ration to readjust to a normal blood stream. The remaining six dogs (No. 300–305) were recently weaned littermates of a heavy breed. They were twice treated with tetrachlorethylene and vaccinated against distemper before being placed on the synthetic diet.

Two synthetic rations,[1] differing only in the quantity of cod liver and cottonseed oil, were used. Ration I contained: sucrose 66, casein (acid washed) 19, cottonseed oil 8, cod liver oil 3, and salts IV 4 per cent; Ration II, sucrose 66, casein (acid washed) 19, cottonseed oil 11, and salts IV 4 per cent. These rations were supplemented with the following crystalline vitamins, riboflavin 100 μg., pyridoxin 60 μg., calcium pantothenate 500 μg., niacin 2 mg., and choline 50 mg. per kilogram of body weight per day. Ration II was further supplemented with 3 drops of haliver oil per kilogram of body weight per week. All vitamin solutions were fed orally

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[1] We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the generous supply of crystalline B vitamins, and to Abbott Laboratories, North Chicago, Illinois, for the generous supply of Haliver Oil.

twice weekly by pipette. Ration I was fed to the adult dogs, Ration II to the littermate pups.

The rations were analyzed for thiamine, and found to contain approximately 0.12 μg. per g., or to contribute approximately 3 μg. per kilogram per day to a 10 kilogram dog eating 250 g. of ration.

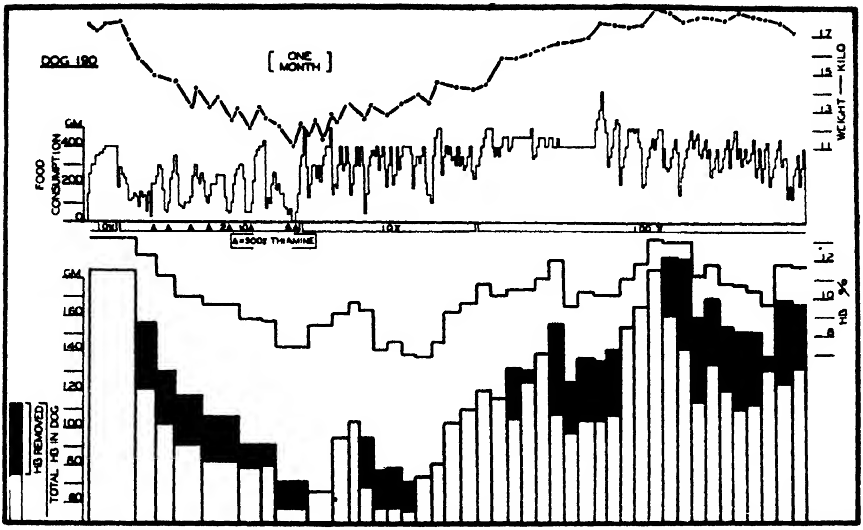
The removal of the blood samples and their analysis for hemoglobin, hematocrit, and cellular counts have been previously discussed (1). In subjecting the adult dogs to the strain of continued bleeding, approximately 25 per cent of the estimated blood volume was removed at a single bleeding. In no instance did this bleeding lower the hemoglobin level of the animal below 7.0 g. per 100 cc. Thirty to 33 per cent of the estimated blood volume was removed at each bleeding in the case of the young dogs until a hemoglobin level of 5.8–6.6 g. per cent was reached. Thereafter, blood removals from the puppies were restricted to the 3 cc. analysis samples. The animals were always bled from the external jugular vein. The volume of blood removed was measured and the hemoglobin concentration determined in order to calculate the amount of hemoglobin removed. The total blood volume of the animal was estimated at 8 per cent of the body weight.

After the four weeks on the complete ration, the five adult dogs showed a normal blood picture. Regular thiamine supplementation was then omitted, thiamine being administered at intervals to combat inanition. At the same time phlebotomy was instituted. The combination of phlebotomy and thiamine restriction was continued until the animals collapsed (90 to 100 days). Glucose-saline solution and 3 mg. of thiamine were then injected, bringing about an immediate and complete recovery. With alternate periods of phlebotomy and rest, the reaction of the animal and the rate of hemoglobin regeneration were investigated on various thiamine levels. The experimental details for a typical adult dog (No. 190) are given in Graph 1.

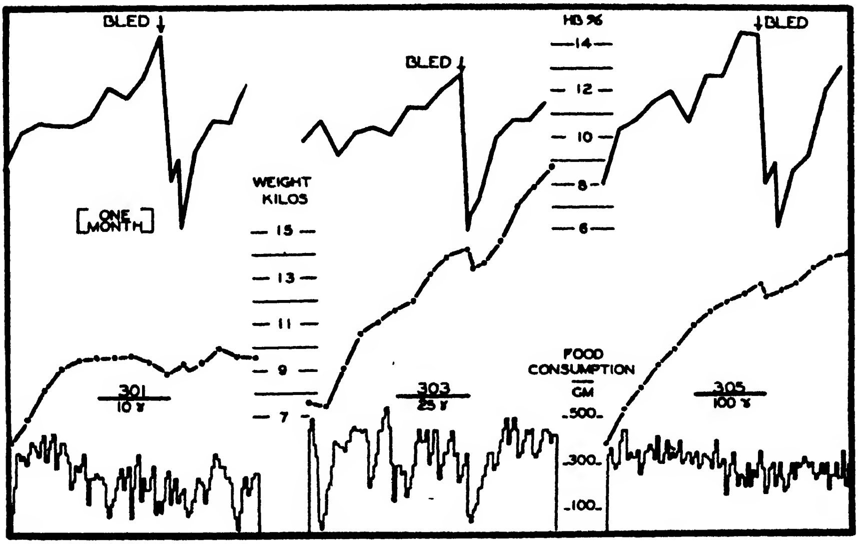
The positive control of the littermate puppies was carefully chosen to represent the average of the litter both in weight and weight gain. The litter was then segregated into pairs to approximate the average weight among each pair of animals. Dogs 300, 301 were maintained on 10 μg., Dogs 302, 303 on 25 μg., Dog 304 on 50 μg. and Dog 305, the positive control, on 100 μg. thiamine per kilogram of body weight per day.

After a period of nine weeks on the synthetic ration, 130 days after birth, the hemoglobin level in the blood of most of the dogs had reached a level approximating that of adult dogs. Dogs 302 and 304 were the exception, and had hemoglobin levels of 11.9 and 11.5 g. per cent respectively. Dog 302 had maintained this level for a period of 7 weeks and Dog 304 for a period of 3 weeks. The remainder of the litter had hemoglobin levels of 12.6, 14.4, 14.4, and 14.8 g. per cent.

At this time the animals were rapidly bled. Dogs 303 and 304 were bled down to the anemic level of 6.0 and 5.8 g. per cent within 3 days. The other dogs of the litter were bled more slowly (8 days) to 6.0–6.6 g. per cent. Phlebotomy was then discontinued, and the dogs were allowed



GRAPH 1
Experimental History of a Typical Adult Dog (190)
The supplementations of thiamine are given as μg. per kilogram body weight per day.



GRAPH 2
The Daily Food Consumption, Weight Gains, and Changes in Hemoglobin g. per cent for Three Growing Dogs

to recover over a period of 27 to 32 days. The detailed results, pictured in Graph 2, show the daily food consumption, weight gain, and changes in hemoglobin level for 3 representative growing dogs.

Adult Dogs

Inanition and loss of weight are the two outstanding symptoms of thiamine restriction. The intermittent administration of 500 μg. doses, an average of 7–10 μg. of thiamine per kilogram per day throughout this period, permitted survival of the animal even under the strain of bleeding. Each supplementation of thiamine brought about a sharp but transitory rise in food consumption with a concomitant rise in weight. There was no observable change in the concentration of hemoglobin. The removal of 10 to 35 g. of hemoglobin at each bleeding was more than the animal was able to regenerate before being bled again, and hence, the animal slowly became anemic. Calculation of the rate of hematopoiesis showed a slightly slower rate of regeneration during this period, especially just prior to the paralysis exhibited by the animal. This lowered rate of hematopoiesis correlated with a sharp drop in food consumption. After collapse, the animals on higher levels of thiamine supplementation were bled again, more hemoglobin being removed at each bleeding than could be regenerated. Cessation of this bleeding caused a rapid rise in hemoglobin in all dogs receiving more than 10 μg. per kilogram per day. Calculation of the rates of regeneration showed that there was little difference for the levels of intake between 10 and 100 μg. per kilogram. Maintenance of body weight or actual gain in body weight was shown by all animals on levels above 10 μg. per kilogram.

These values are slightly higher than the requirements determined by Cowgill (2), 6 μg. per kilogram per day, and Street, *et al.* (3), 6.7–9.0 μg. per kilogram per day, for animal maintenance especially when considering the small amount of vitamins contained in the basal ration. The higher values obtained in this laboratory are probably due to the added strain of phlebotomy that was placed on these dogs.

Growing Dogs

The pair of growing dogs receiving 10 μg. thiamine per kilogram per day consumed an average of 250 g. of ration per day. This food consumption, though erratic, was adequate for some increase in weight.

The average gain in weight by these dogs was 400 g. per week. The dogs receiving 25 μg. of thiamine or more consumed an average of 290 g. of the ration and showed an average growth of 600 g. per week. There was no significant difference in either weight gains or food consumption at the higher levels of thiamine supplementation. These data would place the preferential minimum level of thiamine for the growing dog between 10 and 25 μg. per kilogram per day. Previous work by Arnold and Elvehjem (4, 5) showed that dogs grew normally on diets containing 80 μg. per 100 g. of ration or approximately 20–30 μg. thiamine per kilogram per day. However, no differences in the rate of hematopoiesis could be found comparing the low level of thiamine administration and the higher supplementation levels.

Conclusion

Thus it appears that thiamine has no specific effect on blood regeneration. Though the possibility of a relationship between inanition and decreased blood regeneration can be suggested with 10 μg. of thiamine or less per kilogram per day there is no disturbance of the hematopoietic function either with anemia or under the strain of continued phlebotomy at non-anemic hemoglobin levels with thiamine supplementation above this level. Sufficient thiamine for the maintenance of weight in an adult dog, or for adequate growth in a young dog is sufficient for the maintenance of a normal blood stream even under adverse conditions.

Summary

Growing and adult dogs were maintained on a highly purified ration supplemented with the crystalline B vitamins, exclusive of thiamine. Blood Analyses were carried out at various levels of thiamine feeding with and without phlebotomy, and the rate of regeneration was followed. Anemia was induced in all dogs by phlebotomy.

Food consumption is spasmodic with a thiamine deficiency. Less than 10 μg. of thiamine per kilogram of body weight per day is inadequate for maintenance of body weight for adult dogs. Growth increases at this level for growing dogs are not quite comparable to the increases obtained by littermates on higher thiamine levels.

Adult dogs on a thiamine restricted diet and young growing dogs supplemented with 10 μg. thiamine per kilogram per day show no indication of an anemia.

Inanition, resultant of the thiamine deficiency, when associated with the strain of phlebotomy results in some limiting of the hematopoietic ability of the animal.

There is no disturbance of the hematopoietic function of either growing or adult dogs under the strain of phlebotomy and anemia on a restricted thiamine intake.

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The Tyrosine-Tyrosinase Reaction and Aerobic Plant Respiration

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Introduction

Boswell and Whiting (1) and more recently Baker and Nelson (2) have shown that the phenol oxidase, tyrosinase,[1] functions as the terminal oxidase in a respiratory chain in respiring potato slices (*Solanum tuberosum*), much in the same manner as cytochrome oxidase is supposed to function in certain respiratory systems of many plants and animals (3).

In attempting to ascertain the chemical nature of the natural substrate for the potato oxidase, Boswell and Whiting used the method previously described by Onslow (4). A hot alcoholic extract of potato tubers was treated with lead acetate, the resulting precipitate was freed of lead by means of sulfuric acid, and finally by concentrating the resulting clear liquid a yellow gum like product was obtained. On treatment with ether the gum gave two fractions, one soluble and the other insoluble in ether. Adding an aqueous solution of the latter fraction to some potato slices, respiring aerobically in an aqueous phosphate solution, increased the rate of respiration, indicating therefore the presence of the natural substrate. The product, however, was not identified.

Experiments and Discussion

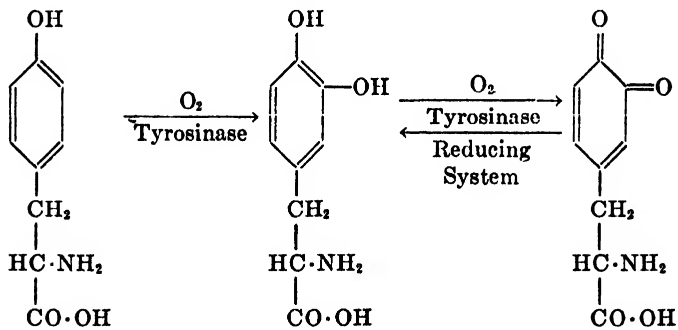
In the present study 50 pounds of potato tubers were passed through a meat grinder and immediately mixed with an aqueous solution of lead acetate and the mixture heated to 60°C. On filtering, the filtrate, after removing the excess lead ion by means of hydrogen sulfide, was found to contain a product which oxidized by means of tyrosinase. On further purification this substance proved to be *l*-tyrosine.

The presence of tyrosine in potato tubers has been known for some time. As early as 1879, Schultze (5) reported the presence of the amino acid in potatoes.

[1] Boswell and Whiting prefer the names polyphenol oxidase or catechol oxidase instead of tyrosinase.

Isherwood (6) mentions the presence of *l*-tyrosine in potatoes, as do Neuberger and Sanger (7). Recently Schmalfuss and Bumbacher (8) also found *l*-tyrosine in potatoes and claim it is the fore-runner of the darkening often noticed when peeled potatoes are exposed to air.

As a terminal oxidase in an aerobic respiration chain, tyrosinase differs from cytochrome oxidase in that it possesses two kinds of enzymatic activity. It not only catalyzes the oxidation of many *o*-dihydric phenols to their corresponding quinones, but it also converts certain monohydric phenols into their respective *o*-dihydric forms. The natural substrate for a terminal oxidase, must have, as its adjacent hydrogen carrier, a substance capable of reversible oxidation-reduction. This is obviously not the case with *l*-tyrosine, because once the second hydroxyl group has been inserted, the 3,4-dihydroxyphenyl alanine (dopa), which is formed, is not capable of direct reduction back to tyrosine. On the other hand, the 3,4-dihydroxyphenyl alanine, formed in the oxidation of the tyrosine, being an *o*-dihydric phenol, and therefore also oxidized by means of tyrosinase, can, in the presence of a suitable reducing agent, function as a reversibly oxidized-reduced hydrogen carrier.



In the light of this limitation of the *l*-tyrosine in not being capable of functioning as a hydrogen carrier, it follows that it must serve as a fore-runner or precursor for the *o*-dihydroxyphenyl alanine, the actual hydrogen carrier adjacent to the tyrosinase in this particular respiratory system.

It has been shown by Raper (9) that when tyrosinase acts on tyrosine in the absence of a reducing system, the reaction does not stop at the corresponding *o*-quinone stage but continues on until a melanin like product is formed. The essential difference between allowing purified tyrosinase to act on tyrosine and the tyrosine-tyrosinase reaction in the potato

cell, is that in the potato cell there is a reducing system present. Therefore, to study the oxidation of tyrosine by means of the enzyme under conditions similar to those in the plant, it was decided to follow the enzymatic oxidation of *l*-tyrosine in the presence of a reducing agent. For this purpose a mixture consisting of *l*-tyrosine, tyrosinase and the reducing agent, ascorbic acid, was permitted to react in the presence of air in a Warburg respirometer. Curve I in Fig. 1 represents the rate of oxygen uptake when 1 mg. of *l*-tyrosine was oxidized by means of tyrosinase in the presence of 2 mg. of ascorbic acid. When the reaction had progressed to the point at which 120 cu. mm. of oxygen had been consumed (Point A on the curve), a pink color appeared indicating the presence of quinoid product in the reaction mixture. Since ascorbic acid reduces quinones, the presence of quinone showed that at this stage in the reaction all of the ascorbic acid had been oxidized. The enzyme had been shown to be inactive toward ascorbic acid, and therefore the latter must have been oxidized by a product resulting from the enzymatic oxidation of the tyrosine. The fact that 127 cu. mm. of oxygen correspond to the amount of oxygen required to oxidize 2 mg. of ascorbic acid, shows that only a trace of tyrosine could have been oxidized, while ascorbic acid remained in the reaction mixture. In other words, the portion of Curve I beyond Point A, represents the rate and amount of oxygen uptake when 1 mg. of tyrosine was aerobically oxidized to the final melanin state of oxidation. The latter claim is supported by Curve II. Curve II represents the rate of oxygen uptake when 1 mg. of *l*-tyrosine was oxidized, under conditions similar to those of the reaction represented by Curve I, except that no ascorbic acid was present. The coordinates were shifted so that the origin coincided with Point A. It will be observed that Curve II practically coincides with Curve I, again indicating that hardly any tyrosine had been oxidized, in the reaction represented by Curve I, as long as any ascorbic acid remained in the reaction mixture.

The fact that only a trace of the *l*-tyrosine was oxidized in the presence of the ascorbic acid indicates that the 3,4-dihydroxyphenyl alanine formed possesses a much greater tendency to combine with the enzyme than does the *l*-tyrosine, and thus prevents the latter from reacting with the enzyme. As soon as the dihydroxyphenyl alanine is oxidized to the quinone the latter is immediately reduced back to the dihydroxy compound by the ascorbic acid. In other words, this small amount of the dihydroxyphenyl alanine serves as a shuttle in transporting, by aid of

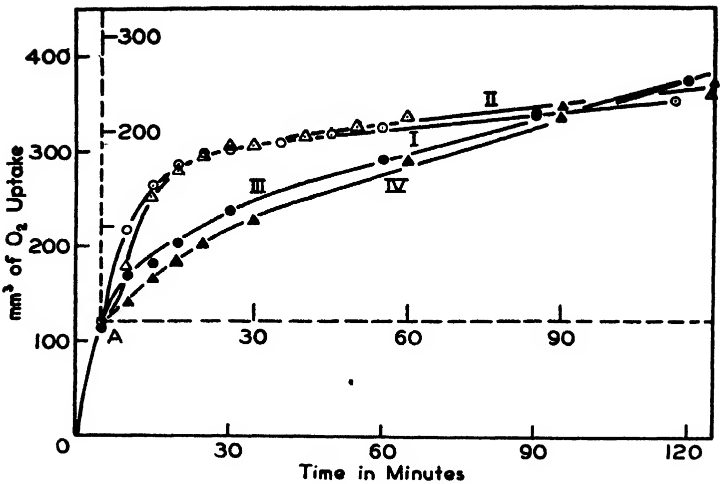


Fig. 1

Showing that *l*-Tyrosine is not Appreciably Oxidized by Tyrosinase in the Presence of *l*-Ascorbic Acid

Warburg respirometer used. Temperature 25° C. and pH 6.1

Curve I: Reaction mixture, 2 cc. 0.2 *M* sodium pyrophosphate, 1 cc. aq. solution containing 1 mg. of *l*-tyrosine, 1 cc. of a solution containing 2 mg. of *l*-ascorbic acid, 1 cc. of a mushroom tyrosinase preparation containing 1.9 tyrosine units[2] per cc. and sufficient water (distilled in glass) to make the final volume equal to 8 cc.

Curve II: Reaction mixture same as for Curve I, except instead of 1 cc. of the ascorbic acid solution, 1 cc. of water was used. The coordinates differ from those of Curve I in that they were shifted so that Point A was the origin.

Curve III: Reaction mixture same as for Curve I, except 1 cc. of a crude potato tyrosinase preparation (0.4 tyrosine units per cc.) was used in place of the 1 cc. of mushroom tyrosinase solution.

Curve IV: Reaction mixture same as for Curve III, except 1 cc. of water was used instead of the 1 cc. of ascorbic acid solution. The coordinates for Curve IV differ from those of Curve III in being shifted so that the origin coincided with Point A.

All reagents except the enzyme solutions were placed in the reaction chamber (50 cc. capacity) of the Warburg respirometer flasks. The enzyme solutions were added from the side-arm to the reaction mixtures after a 10 minute equilibration period at 25°C. The manometers were shaken at the rate of 140 oscillations per minute and the rate of oxygen uptake was found independent of the rate of shaking. Experiments similar to those represented by Curves I and II were performed in which 4 and 8, instead of 2 mg. of ascorbic acid were used. The only difference observed was Point A being raised to about 250 and 500 cu. mm. of oxygen uptake respectively.

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[2] Tyrosine units were determined according to the method used by Adams and Nelson (10) for determining cresolase units. One tyrosine unit corresponds to the amount of tyrosinase required to catalyze the oxidation of *l*-tyrosine at the rate of 10 cu. mm. of oxygen uptake per minute.

the enzyme, oxygen to the ascorbic acid. As soon, however, as the supply of ascorbic acid becomes exhausted, and the quinone is no longer reduced, then the latter leaves the enzyme's surface, and the enzyme is set free to react with the tyrosine and catalyze its oxidation.

Having on hand some highly purified tyrosinase, prepared from the common mushroom, *Psalliota campestris,* it was used in the reactions represented by Curves I and II. Curves III and IV represent reactions similar to those corresponding to Curves I and II, except that a less purified potato tyrosinase was used. Since the potato enzyme preparation was lower in activity toward tyrosine than the mushroom tyrosinase preparation, the rates of oxygen uptake were less than the rates shown in Curves I and II. Nevertheless, beyond the point A at which all the ascorbic acid had been oxidized, Curve III parallels Curve IV representing the oxidation of the *l*-tyrosine in the absence of ascorbic acid. Therefore, here again, just as in the reactions catalyzed by the mushroom tyrosinase, only a trace of the *l*-tyrosine was oxidized as long as any ascorbic acid remained in the reaction mixture.

To show that *l*-tyrosine can take part in the respiratory system of the potato tuber, 50 wet potato slices (1 centimeter square and about 400 $m\mu$ in thickness) (1, 2), previously washed in running tap water for 24 hours, were placed in the reaction vessel of a Warburg respirometer together with 6 cc. of 0.05 *M* phosphate buffer (pH 6.1 and Temp. 25°). In the side arm of the reaction vessel were placed 2 cc. of a slightly alkaline aqueous solution containing 2 mg. of *l*-tyrosine. Part A of the curve in Fig. 2 represents the rates of oxygen uptake and carbon dioxide evolved, caused by the respiring slices before the addition of the tyrosine. The carbon dioxide was determined by Warburg's direct method (11) using filter paper moistened with 0.2 cc. of 20 per cent KOH solution. Since the two rates were practically the same the R.Q. was close to unity. After the slices in the Warburg vessel had been permitted to respire for 30 minutes the tyrosine was added from the side arm. During the first 20 minutes after the addition of the tyrosine, erratic rates of oxygen uptake and carbon dioxide evolution occurred and no readings were recorded. After this the respiration of the slices settled down and both rates again became quite constant (Part B), but greater than before the addition of the tyrosine. This increased rate of respiration, due to the added tyrosine, together with the fact that the R.Q. still continued close to unity, indicates that the added tyrosine took part in the respiration of the potato slices.

Since, as stated above, the potato tuber contains free *l*-tyrosine, the question may arise: Why does the addition of more tyrosine to the respiring slices increase the rate of respiration? It will be recalled that the potato slices had been washed in running water for over 24 hours before they were placed in the Warburg respirometer. During this

time very likely considerable free tyrosine as well as dihydroxyphenyl alanine, originally present, was lost through dialysis. The addition of tyrosine would therefore make up for some of this loss of tyrosine and bring about the observed increase in the rate of respiration.

The fact that *l*-tyrosine, in the presence of a reducing agent, is only oxidized to a slight extent, suggests why considerable free tyrosine can exist in the potato tuber without being oxidized, even though the enzyme is also present. The 3,4-dihydroxyphenyl alanine is the hydrogen

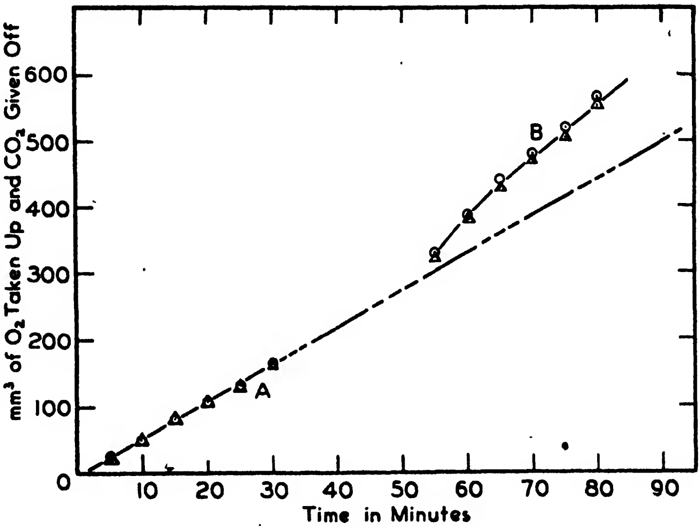

FIG. 2

Showing the Rates of Oxygen Uptake and Carbon Dioxide Given Off by 50 Slices of Potato Tuber Respiring Under Conditions Described in the Text
Circles represent oxygen uptake and triangles carbon dioxide readings
Capacity of the Warburg respirometer flasks was close to 50 cc.

carrier adjacent to the terminal tyrosinase. The *l*-tyrosine serves as a reservoir for keeping the potato cells supplied with the hydrogen carrier. The oxidation of the *l*-tyrosine to the dihydroxyphenyl alanine is, as far as the authors are aware, the first instance in which it has been possible to suggest a physiological rôle for the monophenolase activity of tyrosinase.

The dark coloration observed, when peeled potatoes or the internal potato tissue has been exposed to air, is very likely due to an interruption of the reducing system necessary for keeping the hydrogen carrier, the

dihydroxyphenyl alanine, continuously in the reduced form. This view is supported by the experiments, mentioned earlier, in which it was shown that when the *l*-tyrosine was oxidized by means of tyrosinase, in the absence of ascorbic acid, the oxidation continued until all the amino acid had been converted into the black melanin like product.

Summary

1. It has been shown that only a trace of *l*-tyrosine can be oxidized by means of tyrosinase, when a reducing agent, such as *l*-ascorbic acid, is present in the reaction mixture.

2. It has been suggested that the 3,4-dihydroxyphenyl alanine, the first product formed in the enzymatic oxidation of *l*-tyrosine, acts as a competitive inhibitor in the oxidation of *l*-tyrosine by means of tyrosinase.

3. Data were obtained indicating that 3,4-dihydroxyphenyl alanine is the hydrogen carrier functioning adjacent to the terminal oxidase, tyrosinase, in a respiratory chain present in potato tubers.

4. It is suggested that the free *l*-tyrosine present in potato tubers serves as a reservoir for keeping the potato cells supplied with the hydrogen carrier, 3,4-dihydroxyphenyl alanine.

5. A physiological rôle has been suggested for the monophenolase activity of tyrosinase.

6. The darkening of the tissues of potato tubers, when exposed to air, is attributed to dislocation or interruption of the reducing system in the potato cells.

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A Strain of Shigella paradysenteriae (Flexner) Requiring Uracil

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Introduction

During an investigation of the nutritional requirements of a collection of 20 strains of *Shigella paradysenteriae* (Flexner), Weil and Black (1) found that one of these strains did not grow in any of the synthetic media adequate for the others. Dr. Weil kindly made this strain available to us for further study, and uracil was found to satisfy the additional growth requirement. The simplicity of the other growth essentials favored the possibility of this organism serving as a tool in the study of the metabolism of uracil and of possibly other pyrimidines, and therefore the uracil requirement was studied in some detail.

Experimental

Dr. Weil informs us that the strain, 63-143-V in his collection, is a typical Flexner strain whose main antigen is identical with the race V of Andrewes (Flexner I Boyd).

Cultures were maintained on peptone-agar slants or in semi-solid media, and after incubation 10–24 hours at 35°C. were stored at 4–6°C. Experimental media were distributed in 10 ml. amounts in 25 or 50 ml. Erlenmeyer flasks covered with glass beakers, and were sterilized by autoclaving for 15 minutes at 120°C. Each flask was inoculatd with one drop of a suspension of some of the slant growth in 10 ml. of the basal medium lacking the nutrient in question. Growth appeared indefinitely subculturable in any of the synthetic media found adequate for the first transfer. No unaccountable carry-over effects were observed. Growth was measured after 72 hours. The cultures were killed for this purpose by steaming for 15 minutes at 100°C. Growth was expressed in terms of optical density as determined with a photoelectric densitometer calibrated to read in units proportional to the number of bacteria present. The preparation of the solutions mentioned later, and the details of the instrument, were described in an earlier communication (2).

It was found convenient to supply indole as a 1 per cent solution in 95 per cent ethanol. This solution kept well in the refrigerator and the indole dissolved immediately in the culture medium when added in this form.

Results and Discussion

Besides uracil, the organism required tryptophan and nicotinic acid (replaceable by nicotinamide). These requirements were readily ascertained by use of a series of "screening" media of graded complexity that had been prepared to facilitate the identification of the growth essentials of a miscellany of microorganisms. The organism grew in a medium containing gelatin hydrolyzate supplemented with tryptophan and an arbitrary mixture of the usual growth factors, including uracil. By progressive simplification of the medium it was found that uracil and nicotinic acid were indispensable, and that tryptophan was the only indispensable amino acid. Glutamate and aspartate were helpful, but in the presence of an adequate energy source such as glucose, and nitrate as a hydrogen acceptor, growth, while slower in the absence of these amino acids, reached the same level. Growth was increased by using a shallower depth of medium (secured by substituting 50 ml. for 25 ml. flasks), and by supplying nitrate. Under these conditions growth was not bettered consistently by additions of gelatin hydrolyzate or small amounts of peptone, yeast extract, or urine. Since oxygen (or an equivalent hydrogen acceptor) appears to be one of the factors limiting growth, the sedimentation of the organism may explain in part the difficulty experienced in obtaining smooth growth curves in less than 48 hours. The medium used for the uracil response plotted in Fig. 1 is given in Table I.

The relatively expensive natural tryptophan could be replaced without decrease in efficiency with indole. This is in accord with the findings of Fildes (3) for typhoid bacteria. Anthranilic acid, which was reported to satisfy the tryptophan requirement of certain lactic acid bacteria (4), was ineffective for the Flexner strain. Unfortunately the use of indole has a disadvantage: it forms highly-colored reaction products with the nitrite present as a result of the reduction of nitrate. Efforts to find a satisfactory substitute for nitrate were unsuccessful. Among the compounds tested were those listed by Knight (5) as occasionally efficacious (fumarate, malate, aspartate, asparagine), and also high concentrations of gelatin and casein hydrolyzates.

Uracil did not stimulate the growth of strain X-S 45 of *Shigella paradysenteriae* (Flexner) which requires nicotinic acid but not tryptophan.

As neither of the Flexner strains grew in the absence of nicotinic acid (or nicotinamide), it could not be decided whether fermentable sugars were inhibitory in the absence of nicotinic acid, as reported by Kligler and colleagues (6).

It is interesting to note that for the 19 other strains studied by Weil and Black (1), one grew with ammonium salts as sole source of nitrogen, 15 required nicotinic acid, 3 required both nicotinic acid and pantothenate, and none required tryptophan.

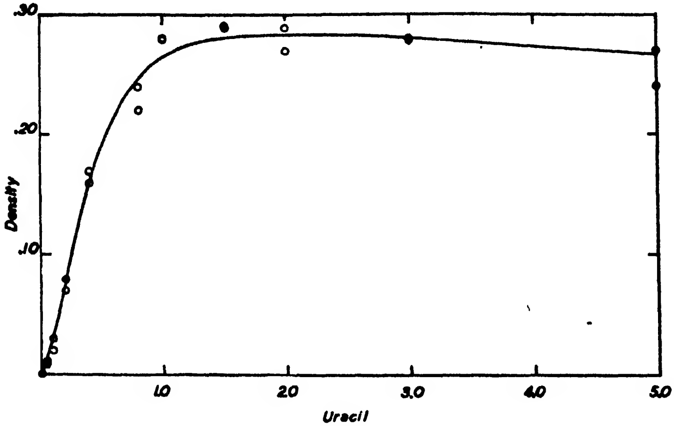

FIG. 1

Growth of *Shigella paradysenteriae* as a Function of Uracil Concentration (Expressed as uracil in mgs. % *vs.* optical density units)

TABLE I

Uracil-deficient Medium for Shigella paradysenteriae

$(NH_4)_2HPO_4$	0.02 g.
K_2HPO_4	0.05 g.
$MgSO_4 \cdot 7H_2O$	0.02 g.
$NaNO_3$	0.2 g.
Glucose (added separately)	0.5 g.
l-Tryptophan	0.01 g.
Nicotinic acid	0.2 mg.
Gelatin hydrolyzate	1.0 g.

Fe 10 μg, Zn 5.0 μg, Ca 10 μg, Cu 1.0 μg, Mn 1.0 μg,
Mo 1.0 μg, B 0.5 μg, I 0.1 μg.
H_2O to 100 ml. pH adjusted to 7.4–7.6.

Use of the organism as a reagent for uracil. Uracil deficiencies ranging in character from an absolute requirement to a stimulatory factor have been reported for five other groups of protists. Uracil was first reported as an absolute requirement of *Staphylococcus aureus* when grown anaerobically (7). It appears to be an absolute requirement of certain strains of

Streptococcus pyogenes (8, 9); a stimulatory factor for *Streptococcus salivarius* (10, 11); a stimulatory factor for certain species of *Lactobacillus* (12), with a consequent extensive use as an ingredient of media for microbiological vitamin assays. Uracil has recently been described as essential for *Clostridium tetani* (13). All these bacteria require either complex media or special conditions of incubation. The simplicity, on the other hand, of media for the Flexner organism would favor the choice of this strain for studies of pyrimidine metabolism. The uracil requirement appears unaffected by wide variations in the composition of the medium provided the other growth requirements are satisfied. Other pyrimidines were not available for a study of the specificity of uracil.

It must be borne in mind that the strain studied here is a pathogen and should be used only by those accustomed to the precautions demanded for work with infectious bacteria.

Summary

An unusually exacting strain of the Flexner dysentery bacterium was found to require uracil in addition to nicotinic acid (or nicotinamide) and tryptophan. Tryptophan was replaceable by indole but not by anthranilic acid. The ease in demonstrating the uracil requirement may recommend this organism as a tool for the study of pyrimidine metabolism.

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BOOK REVIEWS

Dictionary of Biochemistry. Edited by WILLIAM MARIAS MALISOFF, Professor of Biochemistry at Polytechnic Institute of Brooklyn. Philosophical Library, N. Y., 1943. 579 pp. Price $7.50.

There are some obvious faults in this volume. The binding, the paper, the print—the general "get-up" of the book is bad. The diagrams in a number of instances are so small as to be completely illegible. Take, for example, figures 1 and 3, pages 60, 65: they are worse than useless; they are irritating. Or consider the classification of enzymes on pages 222–223; how could an editor sanction such exhibits!

A number of articles, I notice, do not represent specific contributions to the volume under review, but have already appeared elsewhere. This applies, for example, to the sections on amino acids by Van Slyke (p. 22) and immunochemistry by Landsteiner (p. 314).

Some of the definitions leave much to be desired. One or two such examples are acetylcholine (p. 9), anaphylaxis (p. 31) and isoelectric point (p. 325). On p. 18 we find in heavy print "aldehyde oxides" when "aldehyde oxidase" is meant. The article on "acid-base balance of blood" (p. 84) is much too brief and scattered to have any meaning. Much the same criticism applies to the section on "histochemistry" (p. 305).

The impression one gets is that the editor had to evaluate too much material in too short a time. The job appears a hurried and an uncritical one.

However, despite these criticisms, there is still much to be thankful for, due to some fine contributions by different men throughout the country. I refer to a few of these more or less at random: autolysis, p. 40 (Bradley); bacteriophage, p. 57 (Krueger); bioluminescence, p. 75 (Harvey); carbohydrate and fat catabolism, p. 96 (Witzemann); cellulose decomposition by microorganisms, p. 125 (Norman); creatine and creatinine metabolism, p. 150 (Beard); enzymes, p. 212 (Glick); growth, p. 278 (Davenport); hair, p. 288 (Danforth); phosphate-bond energy, p. 403 (Emerson); plant growth hormones, p. 416 (Thimann); protoplasm, p. 440 (Seifritz); teeth, p. 517 (Armstrong and Barnum); wound healing, p. 562 (Arey).

The book represents a pioneer effort, and for this Dr. Malisoff deserves credit. Upon such a foundation Dr. Malisoff, or some other editor, may build at some future time to produce a work of more permanent value.

BENJAMIN HARROW, New York, N. Y.

Physical Biochemistry. BY HENRY B. BULL, Associate Professor of Physiological Chemistry in the Medical School of Northwestern University. John Wiley and Sons, Inc., New York, N. Y., 1943. iv + 347 pp. Price $3.75.

This is the latest of an increasing list of texts that aim to give students of bio-

logy and medicine a background of physical chemistry. The book is an outgrowth of a series of lectures that the author has given to graduate students in biochemistry, physiology, bacteriology, and neurology and to some medical students. The following are the chapter headings: Atoms and molecules, energetics, reaction kinetics, electrostatics and dielectrics, ions in solution, electromotive force cells, acids and bases, oxidation-reduction, electrical conductance, electrokinetics, surface activity, colloidal solutions, viscosity and the flow of liquids, diffusion, the ultracentrifuge, osmotic pressure, membranes and cell penetration, and colloidal structures.

There is no question that in order to cope with the present day problems in biology, students must have an adequate preparation in mathematics, physical chemistry, organic chemistry, and thermodynamics in addition to such courses as provide them with the necessary background in the biological sciences. It is not always easy to impress upon beginning students in biology the desirability, in fact the necessity, of having adequate preparation in the exact sciences if they expect to view biology as a dynamic rather than a static subject. Hence all texts that aim to provide students with some knowledge of the physical sciences, especially physical chemistry, are worth while. Since these texts are not exhaustive in their treatment of the various topics discussed, the more advanced student will of necessity have to consult the more specialized books and monographs and in the end the original literature.

In reviewing the book by Professor Bull the writer has attempted to evaluate it in terms of the student and teacher who will use it rather than as a fault-finding critic. Possibly some of the points mentioned will assist the author in the preparation of a future edition. In order to point out to students the importance of the subject under consideration, it is desirable to present one or more specific examples of the application to biological problems rather than discussing the material in general terms and in the end referring the student to sources where these are given. For example, in discussing the isotopes, one or more specific illustrations of the use of the radioactive and the non-radioactive isotopic elements in the solution of biological problems would have assisted the student materially in grasping the subject. The list of radioactive elements useful to the biologist could easily have been extended. A good deal of work has in recent years been published on the specific rôles of ions and of ion antagonism both in single phase and in multiphase systems. It would have been desirable to present some of these results rather than stating that "ion antagonism is a very complicated subject," and "As ion antagonism is really not a unified phenomenon, very likely it has several causes." While these statements are correct, the student would like to become acquainted with the attempts that have been made to segregate the factors that contribute to the overall phenomenon of ion antagonism.

The treatment of the Henderson-Hasselbalch equation (not mentioned as such) could with justification have been expanded. Practically no discussion is given of the buffers of blood plasma, a subject of the utmost importance to biology students. In discussing isoelectric points of amino acids it would have been helpful to students if the titration curves of typical amino acids had been given. This

would have provided the opportunity of pointing out that the isoelectric points of many of the amino acids are not sharply defined but may extend over several pH units and that a narrow zone is obtained only when the values for pK_1 and pK_2 are less than 4 units apart. The table of amino acid dissociation constants should have included the basic amino acids and the isoelectric point values. It would have been helpful to the student had a distinction between apparent and thermodynamic dissociation constants of amino acids been made.

Some examples of the biologically vital oxidation-reduction systems, for example the coenzymes, would have impressed upon students the importance of oxidation-reduction reactions in life processes. The statement that the potential of the cystine-cysteine system is dependent on the concentration of the reductant and not on that of the oxidant is not thermodynamically sound. It has recently been possible in the writer's laboratory to show that in the presence of potassium iodide the same potential is obtained on cystine-cysteine mixtures as when cysteine is titrated with an iodine solution. The potential depends on the relative concentrations of oxidant and reductant as would be expected in such a system. It would have been helpful to students had some discussion of electrical transport been given. This would have enabled the writer to bring out the distinction between electrophoretic mobility and ionic mobility as obtained from transference data and by extrapolation of equivalent conductance to infinite dilution as well as the mobility of ions determined by diffusion measurements. It should have been pointed out that as a result of denaturation the osmotic pressure of some proteins when dissolved in urea solution is not the same as when these proteins are present in aqueous solution. Data and x-ray photographs of the α- and β-keratin transformation would have made the subject of x-ray diffraction easier for the student to understand. Similarly, electron photomicrographs of virus crystals would have impressed upon students the importance of this new tool to the biologist.

It is heartening to note the statement (p. 214) "that there is no fundamental difference between adsorption and chemical binding; identical types of forces are operating." However, the author apparently cannot get entirely away from the use of the rather indefinite term adsorption since in speaking of proteins (p. 127) he states that "certain ions may be adsorbed from the solution." It would have been helpful to students had it been pointed out that, under certain conditions, the adsorption isotherm formula which is usually taken as a criterion for adsorption can be derived from the mass law and hence constitutes no real criterion for the use of that term. In the light of modern chemical concepts the terms adsorption and colloids have lost most or all of their original meaning and the time will probably come when they will be largely of historical interest.

Professor Bull's book has much to commend it. It should prove very useful in providing students in the biological sciences with an orientation in physical chemistry particularly those aspects that have been applied to the solution of biological problems. It is hoped that in due time the text may be expanded somewhat particularly with respect to applications of physical chemistry to biological problems. A great opportunity is afforded for the twain to meet.

CARL L. A. SCHMIDT, Berkeley, Cal.

Fundamentals of Immunology. By William C. Boyd, Ph.D., Associate Professor of Biochemistry, Boston University, School of Medicine, and Associate Member, Evans Memorial, Massachusetts Memorial Hospitals, Boston, Mass. Interscience Publishers, Inc., New York, N. Y., 1943. xiv + 446 pp. Price $5.50.

By abandoning the customary historical method of approach and basing his presentation on modern concepts, Dr. Boyd has prepared an unusually lucid presentation of the general field of immunology, which should be valuable to the serious student as an easily understandable introduction to the subject and to the immunologist and the immunochemist as a reference book in which the present state of the science is clearly summarized. The Author, while emphasizing basic principles, has included discussions of many practical applications.

The scope of the book may be judged from the chapter headings: I. Immunity and Immunology, 20 pp.; II. Antibodies and Antibody Specificity, 62 pp.; III. Antigens, 30 pp.; IV. Cell Antigens, 24 pp.; V. Blood Groups, 24 pp.; VI Antibody-antigen Reactions, 88 pp.; VII. Complement and Complement Fixation, 20 pp.; VIII. Anaphylaxis and Allergy, 36 pp.; IX. Allergy and Immunity; Bacteria; Viruses, Parasites, 16 pp.; X. Practical Use of Artificial Immunity, 20 pp.; XI. Laboratory and Clinical Technic, 90 pp.

An outstanding feature of the book is the excellent chapter on laboratory and clinical techniques, which are described in considerable detail. This includes the handling of apparatus, injection and bleeding of animals, and preparation and titration of serums and antigens for clinical use. Procedures used in theoretical studies include determinations of optimum proportion and equivalence zones, specific inhibition and absorption, preparation of azoproteins, and statistical analysis of animal titrations. Unfortunately references to some procedures have been omitted.

The Author's interest in the problem of the nature of serological reactions is reflected in the extensive discussion in Chapters II and VI of the pertinent experiments of Landsteiner, Heidelberger, Haurowitz, and others and of the theories now accepted or given serious consideration. This discussion is in general excellent; it is marred, however, by an attitude of pronounced opposition to the lattice or framework theory of agglutination and precipitation, which contrasts markedly with the judicial presentation of other subjects.

The book represents a valuable contribution, particularly to those interested in the theoretical aspects of immunological reactions. The difficulties of devising a text suitable for both biologists and chemists have not been entirely overcome. The superficial treatment of such problems as allergy, cellular reactions, and the role of immune reactions in disease detracts from the usefulness of the book in medical courses. In some instances only one side of the case is presented, as, for example, in the discussion of the differences between allergy of infection and Arthus type of hypersensitivity. Although he makes only brief mention of immunity to animal parasites, the Author is to be commended on his recognition of this much ignored, but important field.

There has been need for a book of this type, and we feel that the present volume meets the need satisfactorily.

Linus Pauling, Pasadena, Cal.
Dan H. Campbell, Pasadena, Cal.

Plants and Vitamins. By W. H. Schopfer, Director of the Botanical Institute, University of Bern. Authorized translation by Norbert L. Noecker, Department of Biology, University of Notre Dame. Chronica Botanica Company, Waltham, Mass. and G. E. Stechert and Co., New York City, 1943. xiv + 293 pp. 3 plates. Price $4.75.

This volume is the first to be devoted solely to questions concerning the physiology of vitamins from the standpoint of the plant. Vitamins are considered in their roles as plant growth factors and plant growth regulators. Professor Schopfer is himself a distinguished figure in the field and was among the first to demonstrate the role played by vitamins in the control of plant growth. That sufficient material has accumulated to justify the appearance of this book is evidence of the striking advances which have been made in the field in the ten years of its existence.

The book is divided into three sections, e.g. 1) vitamins in plants which themselves synthesize the materials, 2) vitamins in plants which are not capable of synthesizing the substances in question, and 3) general problems arising from 1) and 2). Part 1) considers the principal vitamins produced in plants, in particular in higher plants, and then discusses what is known concerning the activity of each material in the plant and the roles played by vitamins in the regulation of the growth of embryos, leaves, isolated roots and other tissues, the rooting of cuttings, and the germination of pollen. This is followed by a summary of the information concerning factors influencing the synthesis of the several vitamins by the plant. In this as in other sections there are occasional errors in the text particularly in the citation of literature. Under part 2), the author takes up the growth factors of microorganisms, including bacteria, the yeasts, filamentous fungi, flagellates and algae. Considerable emphasis is placed on thiamin and organisms requiring thiamin. Part 2) includes also a discussion of the functions of the several vitamins and in particular their roles in coenzymes. Part 3) is concerned with general problems related to vitamins, vitamin cycles in nature, growth factors and sexual expression in plants, growth factors in symbiosis and parasitism, and the use of microorganisms in vitamin assays.

This book will be of great use to students in the field although in this as in any rapidly expanding subject new contributions have already advanced our knowledge beyond the limits indicated by the author. It is to be hoped also that this volume may emphasize to plant physiologists the importance to their field of the approaches of chemical physiology.

James Bonner, Pasadena, Cal.

Biochemistry of the Fatty Acids and their Compounds, the Lipids. By W. R. Bloor, Professor of Biochemistry and Pharmacology in the University of Rochester, Rochester, N. Y. American Chemical Society Monograph No. 93. Reinhold Publishing Corporation, New York, N. Y., 1943. xi + 387 pp. Price $6.00.

As the title indicates, this volume emphasizes the behavior of lipids in the body and the effects of normal and abnormal metabolic activities upon their distribution in tissues. Hence the book fills an important place in supplementing and enlarging upon the recent volumes by Bull (1937) and Hilditch (1940).

The text is divided into six chapters as follows: I, Chemistry, Descriptive and Analytical, 57 pp., 267 references; II, Digestion and Absorption, 57 pp., 289 references; III, Lipids of Blood, 81 pp., 392 references; IV, The Lipids in Tissues, 90 pp., 410 references; V, Lipid Metabolism, 64 pp., 337 references; and VI, The Lipids of Secretions and Excretions, 28 pp., 154 references.

The number of references cited indicates the large amount of work done in the field of fat metabolism. As the author points out in the prefaee, "Limits of space prohibit the listing of all or even the more significant publications. What is given is a list of those articles which either contribute most to the general discussion or contain references which lead the interested reader to other material not specifically mentioned." In spite of this restriction Dr. Bloor has successfully produced a book well balanced in regard to subject matter and has avoided the common tendency of authors to overemphasize those topics in which they are particularly interested. Possibly this is due to his broad personal experience in the field, for which he has done more than any other American, either directly or through his students and associates.

The reviewer is not in complete agreement with the author's interpretation of some data and would put more emphasis on very recent work in summarizing the present status of our knowledge of such topics as fat utilization, essential fatty acids, etc. However, this is a matter of personal opinion and in no way detracts from the value of the book for reference. The subject matter has been subdivided into many sub-topics which aids the reader in finding quickly what he seeks in the anatomical, physiological, or chemical categories. The exhaustive treatment of the early literature makes this book of great value to the specialist in the field of fat biochemistry. It is recommended for immediate addition to his library.

George O. Burr, Minneapolis, Minnesota.

ERRATA

Vol. **3**, p. 441, in **Table I**:
read ***Biotin 0.2 γ***
for ***Biotin 0.2 g.***

The Chemical Composition and Nutritional Value of Pollens Collected by Bees[1]

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Received March 1, 1944

Introduction

The chemical composition and properties and the nutritional value of pollen collected by bees are of considerable importance in a variety of fundamental problems of interest to plant physiologists, plant breeders, physicians, entomologists, and to nutritionists, both animal and human.

Pollen is of special interest to entomologists because it is the chief source of all foodstuffs, except water and carbohydrates, required by the bee. It provides most, if not all, of the fundamental nutrients for making royal jelly, which nourises the queen larvae and also the very young worker larvae. From pollen comes the chemical substances out of which are made the muscles, vital organs, glands, hairs, and wings. It also furnishes materials for the repair of worn out tissues. Pollen is, therefore, essential for the growth of individual bees and to the development and reproduction of colonies. When pollen is brought into the hive by bees it is stored in the cells of the combs where it undergoes a lactic acid fermentation. The fermented pollen is called bee-bread. Apiarists are frequently forced to provide artificial pollen or bee-bread for the nourishment of their colonies.

The nutritional value of pollen, as indicated by its chemical contents of inorganic elements, vitamins, and dietary indispensable amino acids,

[1] Paper No. 2147, Scientific Journal Series, Minnesota Agricultural Experiment Station. The data in this paper were taken from a portion of the thesis submitted by A. Earl Vivino to the Graduate School of the University of Minnesota in June 1943 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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is of interest to nutritionists, not only because of its importance for the rearing of bees, but also because of its possible importance for vertebrate animals and even for humans, since it has been estimated by Todd and Bretherick (1942) that the bees in the United States collect annually 80,000 tons of pollen, an amount comparable to their honey production. There have been no previous investigations from these viewpoints of pollens collected by bees in Minnesota.

EXPERIMENTAL

The chemical composition and nutritional value was determined for pollens collected by bees at the University of Minnesota apiary during the season from the latter part of April through September, 1942. The pollens were secured for study by the use of pollen traps of the type developed by Schaefer and Farrar (1941). The trap consists of a double grid of five-mesh hardware cloth, through which the bees must pass, which scrapes off the pollen pellets from their legs as they enter the hive. These pellets fall through a screen into the pollen trap beneath.

Ten traps were used in the collection throughout the season. The pollen after collection was spread out for partial drying for 24 hours. This reduced the moisture content to about 25 per cent. The samples then were placed in individual closed containers and stored at a temperature of $-20°$ C. They are referred to in this paper as "fresh" pollens. At the end of the season, the pollens were assembled into four major groups, showing the following predominance of certain pollens: dandelion and fruit, fruit, clover, goldenrod and aster. Chemical assays were made on each group of pollen. For biological assay a composite sample of the collection periods was used. Certain assays, *i.e.*, ascorbic acid and riboflavin, were made on the samples at the time of their collection, without submitting them first to partial drying.

The methods of proximate analyses used were those of the Association of Official Agricultural Chemists (1940). Calcium, phosphorus, and magnesium were determined by the methods of Morris, Nelson, and Palmer (1931).

For iron and copper determinations special samples of pollen were used, with precautions to avoid contamination. Available iron was determined by the a-a′-bipyridine method of Kohler, Elvehjem, and Hart (1936). The sodium diethyl dithiocarbamate method of Conn, Johnson, Trebler, and Karpenko (1935) was used for the determination of copper. Thiamine was determined by the method of Hennessy and Cerecedo (1939) as modified by Hennessy (1941). Samples of pollen not partially air dried were analyzed at weekly intervals for ascorbic acid by the photoelectric indophenol method of Bessey (1938). For purposes of comparison the data were eventually recalculated to the same moisture content as the partially dried pollens. The method of Koehn and Sherman (1940), as modified by the Technical Committee for Vitamin A Researches[3], was used for

[3] See Berl, S., and Peterson, W. H., *J. Nutrition* **26**, 527 (1943) for description of this method.

the determination of vitamin A and carotenoids. Microbiological methods were used for riboflavin, pantothenic acid, and nicotinic acid; the methods of Snell and Strong (1939) for riboflavin, of Pennington, Snell, and Williams (1940) for pantothenic acid, and of Snell and Wright (1941) for nicotinic acid. In the assays for vitamins D and E, ether extracts of a composite sample of the pollens were used. The method employed for the assay of vitamin D was that of the United States Pharmacopoeia XI, Second Supplement (1942). Vitamin E was assayed by a modification of the method described by Palmer (1937). The chick assay of Almquist (1941) was used in determining vitamin K. A preliminary study of amino deficiencies of the pollen proteins was made by rat feeding experiments, using the ether extracted mixed pollens as a source of proteins. Qualitative tests were made for chlorophyll, anthocyanins, and flavone pigments. A Cenco Photelometer was used for all colorimetric estimations, employing appropriate standard curves of reference.

The group and seasonal averages of the proximate analyses and of the mineral and vitamin contents of the four groups of pollens and the coefficients of variation of the seasonal averages are shown in Table I (chiefly on the "fresh" basis) together with information regarding the source of the pollens.

Discussion and Conclusions

From the data presented in Table I it is evident that the pollens collected by the bees in this study had a high nutritive value.

The mean concentration of the mineral constituents of the ash of the Minnesota pollens was found to be only slightly different from that found by Todd and Bretherick (1942) for California pollens. However, neither on the basis of the mineral contents nor on the basis of the other components, when considered as a whole, is it possible to classify these pollens with any of the common groups of food or food products of plant origin. The protein, fat, phosphorus, and iron contents resemble most closely dried navy and kidney beans, as well as dried peas and lentils but the pollens analyzed were much richer in calcium and magnesium than is any of these seeds. The high copper content of the pollens is outstanding and no doubt explains the high copper content of certain honeys.

The only reference to the vitamin content of bee-collected pollen in the literature is that of Pearson (1942) who obtained an average of 24.4 γ of pantothenic acid per gram fresh pollen. The bee-collected Minnesota pollen analyses shown in Table I contained an average of 22.0 γ of pantothenic acid per gram with a coefficient of variation of 18.73 per cent. The results of the two studies coincide very closely on a moisture-free basis. Bee-collected pollen is, therefore, one of the rich sources of pantothenic acid. On an equal moisture basis it is exceeded only by beef

brain, eggs, cauliflower, Irish potatoes, and tomatoes and compares favorably with a number of muscle meats[4].

TABLE I

Results of Analyses of Four Groups of Pollen. "Fresh" Basis

	Group I	Group II	Group III	Group IV	Mean	P.E.	C.V. %
Moisture, %	24.22	25.71	21.37	24.25	23.89 ±	0.88	6.45
Dry matter, %	75.78	74.29	78.63	75.75	76.11 ±	0.97	2.22
Ether, %	4.33	4.43	1.43	3.26	3.34 ±	0.72	37.84
Ash, %	2.90	2.24	2.82	2.68	2.66 ±	0.14	9.17
Crude protein, %	19.31	18.01	21.99	21.31	20.15 ±	0.95	8.17
Calcium, %	0.20	0.34	0.41	0.22	0.29 ±	0.06	32.76
Phosphorus, %	0.42	0.41	0.58	0.30	0.43 ±	0.05	20.47
Magnesium, %	0.19	0.27	0.23	0.25	0.24 ±	0.01	11.06
Copper, mg. %	1.40	1.10	2.1	1.7	1.57 ±	0.23	24.83
Iron, mg. %	0.20	0.76	0.63	0.59	0.55 ±	0.11	35.47
Carotenes, γ/g.	47.5	110.1	150.0	72.6	95.0	± 22.45	40.93
Xanthophylls, γ/g.	140.75	321.5	412.5	158.8	258.4	± 65.49	43.89
Nicotinic acid, γ/g.	132.0	197.0	200.0	210.0	184.7	± 17.97	16.85
Pantothenic acid, γ/g.	16.0	22.6	27.6	21.8	22.0	± 2.38	18.73
Thiamine, γ/g.	10.8	6.31	9.28	10.31	9.17 ±	1.02	19.30
Riboflavin, γ/g.	19.2	16.3	18.5		18.5	± 0.57	11.94
Ascorbic acid, γ/g.	152.0	176.0	161.0		159.0	± 0.70	16.98
Vitamin D, Int'l. Units/g. pollen fat							0.2–0.6
Vitamin E, mg./g. pollen fat							0.32
Vitamin K							0.00
Collection period	4/21–5/10	5/16–24	6/9–8/29	8/31–9/25			
Dominant pollen	Dandelion Plum and apple	Plum and apple	Clover (alsike, white and sweet)	Goldenrod and Aster			

The nicotinic acid content of the pollens was found to increase steadily during the summer, as shown in Table I. It is evident that the "fresh" pollens had a concentration of nicotinic acid approaching that reported

[4] cf. Cheldelin, V. H., and Williams, R. J., *Univ. of Texas Pub.* **4237**, 105 (1942).

for fresh beef muscle and liver and exceeded only by wheat bran and yeast[5]. It was much higher than has been reported for the common dried beans and peas.

The thiamine content of the "fresh" pollens was found to be lowest in Group II (from plum and apples) which suggests that dandelion pollen dominated the first group. When compared on an equal moisture basis the mean seasonal value of 9.17 γ/g. exceeds considerably the thiamine content both of the legume seeds having like protein and fat and of any of the whole cereal grains. It approximates more closely (also on an equal moisture basis) the thiamine content of certain cuts of meat (raw), such as beef liver and the edible portion of leg of lamb[6].

The mean concentration of ascorbic acid in the "fresh" pollens was about the same as that reported for raw fresh endive, lettuce, and rhubarb, canned sweet potatoes and steamed white potatoes, or commercial canned tomatoes.[6]

Weekly assays of the riboflavin content of the pollen before partial drying showed a mean concentration of 18.5 γ/g. when recalculated to the same moisture content as the partially air dried ("fresh") pollen. Euler, Burstron, and Larsson (1934) reported that pollen of birch and elm contained 9 γ of "flavin" per gram. With the exception of various strains of yeast no product of plant origin has shown so high a content of riboflavin as was found in the pollens in this study. The concentration resembles that in dried skim milk.

The bee-collected pollens were found to possess some antirachitic activity, as disclosed by U.S.P. assay of the extracted "fat," in an experiment involving 49 rats. There has been some debate as to whether fresh plant materials possess such activity. Hess and Weinstock (1924–5) tested some wheat and lettuce leaves before and after irradiation and found that these substances possessed activity for rats after irradiation. Hess, Weinstock, and Sherman (1925) reported that "lettuce which was grown out of doors during the months of May, June, and July failed to protect rats from rickets. The lettuce was not 'in head' and, therefore, well exposed to the rays of the sun; the leaves were plucked on the day of feeding and given in 10 g. per capita amounts." The failure was attributed to a "lack of intensity of the ultra violet radiations of the sun." Chick and Roscoe (1926) found that spinach grown in the open in the

[5] cf. Hale, E. V., Davis, G. K., and Baldwin, H. R., *J. Biol. Chem.* **146**, 553 (1942).

[6] cf. Hewston, E. M., and Marsh, R., *U. S. Dept. Agr. Misc. Pub.* **505**, (1942).

winter, spring, or autumn possessed no antirachitic properties, but spinach grown in midsummer had a slight but appreciable antirachitic value. Although Hess, Weinstock, and Helman (1925) activated impure phytosterol by irradiation with a quartz mercury vapor lamp, the experiments of Steenbock and Black (1925) with irradiated crystalline phytosterol were inconclusive.

The pollens were probably exposed in the trap to sunlight for as much as 12 hours. Since the pollen "fat" from the mixed pollens collected by the bees contained between 0.2 and 0.6 International Units vitamin D per gram, it is evident that activatable sterols were present and that there was sufficient exposure to the ultra violet rays of the sun to effect some activation in spite of the high moisture content of the product.

The pollen "fat" was also found to possess vitamin E activity. The feeding of 1.75 g. of pollen "fat," in 0.35 g. daily dose, during a five-day period (4th to 8th day of gestation, inclusive) to 10 vitamin-E-free female rats produced a ten per cent fertility rate, while the feeding of 2.50 g. of "fat" under like conditions produced a 90 per cent fertility rate. Ten rats in a negative control group and a like number given 1.25 g. gave zero fertility. From these results the mean fertility dose of pollen "fat" was calculated to be 2.09 g. Kielley (1942), in this laboratory, found that 0.64 mg. of Merck's natural α-tocopherol given to female rats during the same stage of gestation period produced a 50 per cent mean fertility rate. The α-tocopherol equivalent content of the pollen was calculated from this result

The mixed pollens possessed no vitamin K activity. When fed to 10 vitamin-K-free chicks in 10 g. doses, the material did not relieve the chicks of their deficiency, the mean prothrombin time exceeding one hour as contrasted with a mean prothrombin time of 34 seconds for 10 normal chicks and 39 seconds for 5 deficient chicks which had received 2 mg. of the vitamin K standard (2-methyl-1,4-naphthoquinone). Bee-bread, which is fermented pollen, possesses vitamin K activity[7]. It is known that feces, which contain the residues from a large number of bacteria, possess vitamin K activity. Probably the bacteria which accompany or assist in the fermentation of pollen to bee-bread synthesize vitamin K.

Vitamin A, as such, was not detected in the mixed pollens. This has been found to be true for all plant material.

Due to its high content of protein, vitamins, minerals, and fat, it was

[7] Unpublished results by Vivino and Haydak, obtained in this laboratory.

found possible to use "fresh" pollen as the sole source of food for rats. Three rats fed "fresh" pollen as the only food for 29 to 54 days made an average daily gain of 2.6 g. When ether extracted mixed pollens were fed to small groups of rats as the sole source of protein (level of protein being about 22 per cent) in a diet otherwise complete, and their growth compared with others receiving the same ration supplemented with certain of the known dietary indispensable amino acids, indications were obtained of tryptophan, methionine (or cystine) deficiency in the pollen proteins. This experiment was not sufficiently extensive to warrant a detailed description in this paper.

Carotenoids were found in abundance in the pollens. The carotenoids consisted of carotenes and xanthophylls, with mean concentrations of 95 and 258.4 γ/g., respectively. The ratio of xanthophylls to carotenes remained about the same throughout the season. Qualitative tests of the pollens were made for chlorophyll and anthocyanins but none were detected. Flavones or anthoxanthones were found in great abundance.

Summary

Pollens collected by bees from dandelion, certain fruits, clovers, and fall flowers were found to have a high nutritive value. The concentration of the mineral constituents, calcium, phosphorus, magnesium, iron, and copper, compares favorably with a number of plant foods. The copper content was exceptionally high. The pollens proved to be a good source of pantothenic acid, nicotinic acid, thiamine, riboflavin, and ascorbic acid. They also contained small amounts of vitamins D and E. The water-soluble vitamin concentrations of bee-collected pollens were as high as in a number of the better food sources of these respective factors Vitamin K was not present. Vitamin A was also absent.

Pollens collected by bees from the sources mentioned were found to contain an abundance of carotenoids and flavones. Anthocyanins and chlorophylls were absent.

The mean protein content of bee-collected pollens studied was found to compare favorably with that of common beans, peas, and lentils. When the ether extracted mixed pollens were fed to rats as the sole source of protein at about 22 per cent level, in a diet otherwise complete, indications were obtained that certain dietary indispensable amino acids, notably tryptophan and methionine (or cystine) were not present in optimum amount for normal growth of this species.

The authors wish to express their indebtedness to Dr. M. C. Tanquary and Dr. M. H. Haydak, of the Division of Entomology and Economic Zoology, University of Minnesota, for much helpful guidance.

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Phosphorus Metabolism

VIII. Phosphorylation of Glucose and Pentoses by the Kidney Cortex

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Introduction

Solution of the problem of kidney tubule resorption, particularly that of the resorption of carbohydrates, has led to investigations concerning phosphorylation in this organ. Previously Laszt and Sullmann (1) were the first to find an increased amount of phosphate esters in the *intestinal* wall during resorption of hexoses, but not of pentoses. Lundsgaard (2) confirmed this for glucose and fructose. Since that time considerable investigation has been made to establish this finding and to determine if the same holds true for various carbohydrates. The prevailing inference from the available though insufficient data appears to be that, as a rule, hexoses are phosphorylated but not pentoses.

The coupling of oxidation and synthesis of inorganic P to phosphate esters in blood demonstrated in the experiments of Runnstrom and Michaelis (3), previously shown by Runnstrom, Lennerstrand, and Borei (4), led Kalckar (5) to try reverse phosphorylation by the kidney, it being already well known that dephosphorylation was brought about by kidney phosphatase. Kalckar showed that the kidney cortex "is certainly able under certain definite conditions to phosphorylate great amounts of glucose." Later Kalckar (6, 7) discovered the accumulation of phosphoric acid esters in fresh kidney extracts under aerobic conditions in a suitable concentration of fluoride. Colowick, Welch, and Cori (8) confirmed and extended the work of Kalckar, and Beck (9) also found phosphorylation by kidney cortex extracts.

The only work reported on kidney phosphorylation of pentose appears

to be that of Kalckar (5) who states that arabinose is very slowly phosphorylated. He gives no data whatever. Dickens reported (10) some work on free- and phosphorylated pentoses, mainly the latter, with yeast, finding that free pentoses were not fermented; but no work was done on animals. It should be noted, however, that Wirth and Nord (11) were able to show that Fusaria ferment not only hexoses but pentoses, and do not appear to utilize, enzymatically, organic or inorganic phosphates during the first phases of the dissimilation.

That there is resorption of pentoses, at least of xylose, from the kidney tubules of mammals and man has been reported by Walker and Hudson (12), Shannon (13) and Shannon and Smith (14). Other reports on xylose disposal by the kidney deal only with clearance by filtration through the glomerulus or with excretion of this carbohydrate through the tubules into the urine. If the resorption which is reported should be further substantiated it would still remain to prove whether it is by simple diffusion, active secretion, or phosphorylation.

The object of the experiments presented in this paper was to determine if the pentoses: xylose, ribose, and arabinose are phosphorylated like glucose under coupled oxidation-synthesis conditions by extracts of fresh kidney cortex. In order to do this the laboratory technique and determinations to prove glucose phosphorylation were first established, also the extent of the process. The mechanism of carbohydrate phosphorylation was assumed to be that indicated by Kalckar (6, 7), Colowick, Welch, and Cori (8) and as further discussed by Ochoa (15) for heart extracts. Some changes in technique from that of the above authors were made, especially since more analyses were to be made because of longer incubation periods and also since the oxygen determination was to be omitted.

Method

Adult rabbits were used as the source for fresh kidneys. The animals were killed by chloroform-ether anaesthesia or by air thrombosis, the kidneys were immediately removed, the cortex was sliced off and 15 g. were pulped in an ice-cold mortar with a pestle after adding several grams of acid-washed sand. Fifteen ml. of $M/15$ phosphate, pH 8.0, and 15 ml. of water were added and after stirring, the mixture was centrifuged in a 30 ml. conical tube at about 1500 r.p.m. for 30 seconds. The supernatant liquid was poured into a 25 ml. graduate, and 16.7 ml. were pipetted into a 250 ml. Erlenmeyer flask which was to serve as the incubation vessel. This was then kept in a water bath at 39–40° C. Ten ml. of the "reagent solution" (see below) was added and after immediate mixing, 2 ml. (for sugar determination) were pipetted into a vial containing 1.25 ml. of water. One ml. of

a saturated solution of $HgSO_4$ in 10% H_2SO_4 was immediately added while stirring. For the inorganic P determination 1 ml. of the incubation mixture was immediately pipetted into a 10 ml. flask containing 1.25 ml. of 10% trichloroacetic acid. Oxygen was continually supplied to the surface of the reaction mixture from a tank connected by tubes through a 2-hole stopper, and the contents were kept in continual motion in the water bath by means of a small motor mixer. Samples for analyses were pipetted out every half hour or hour.

Glucose or pentose were determined on each sample by the preparation of a filtrate according to West and Peterson (16), followed by the copper reduction procedure of Somogyi (17)[1]. Inorganic P was determined by the colorimetric method of Youngburg and Youngburg (18).

Composition of the Reagent Solution Added to the Kidney Extract

Every 10 ml. contained the following:

10 mg. Muscle adenylic acid
1.12 ml. NaF, 2%
2 ml. Succinic acid (10 ml. = 381 mg.), neutralized
3.8 ml. $MgCl_2$ (1 ml. = 1 mg. Mg)
119 mg. Glucose, or 100 mg. pentose

l-xylose, *d*-ribose, and *l*-arabinose were used. The pentose was in equimolecular amount to glucose.

Composition of the Incubation Mixture

(Proportion: 16.7 ml. kidney extract to 10 ml. of reagent solution)

Every 11.2 ml. (considered as unit volume) contained the following:

2.08 g. Pulped kidney cortex
3.12 ml. Phosphate buffer, *M*/15, pH 8.0, 4.82 mg. of inorganic P
9.4 mg. NaF, 0.02 *M*
32 mg. Succinic acid, 0.024 *M*, as succinate
1.6 mg. Mg^{++}, as $MgCl_2$, 0.0059 *M*
50 mg. Glucose, or 42 mg. pentose

See Method for more strict designation of the quantity.

Results and Comment

Figs. 1A and B gives the results of 10 experiments which were designed to show whether or not glucose phosphorylation by kidney cortex actually took place under our procedure, the extent of such phosphorylation, and the effect of varying certain conditions or factors. Having found the

[1] For equal weights the relative reduction values of the carbohydrates by the method of Somogyi were found to be the following:

Glucose	100
Xylose	93.7
Ribose	80.4
Arabinose	69.9

conditions for abundant glucose phosphorylation we were in a position to determine if pentoses reacted similarly. The results of the latter are given in Fig. 2.

Experiments 1 and 2 show that fresh extracts of rabbit kidney cortex strongly phosphorylate glucose under aerobic conditions, there being a

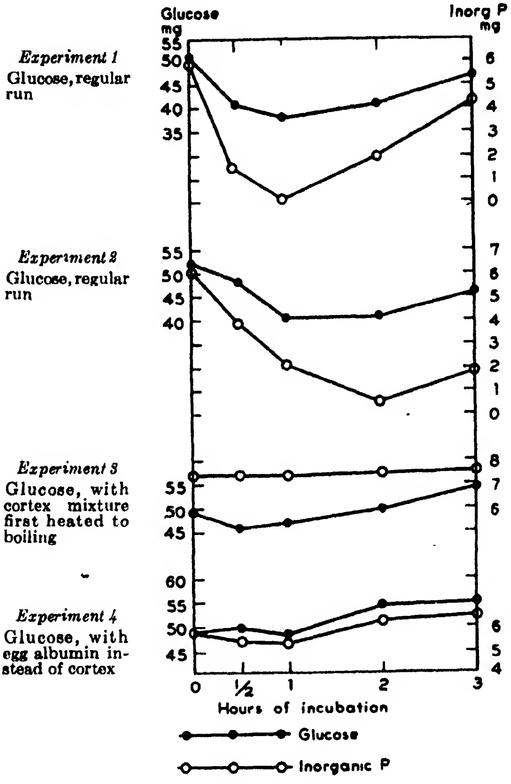

FIG. 1A

FIGS. 1A AND B. Changes in Concentrations of Glucose and Inorganic Phosphate on Incubation with Fresh Rabbit Kidney Cortex.

large decrease in the reduction capacity, and the inorganic P of the reaction mixture greatly decreased. That such phosphorylation does not take place anaerobically is shown in Experiment 10. The work of Kalckar and of Colowick, Welch, and Cori previously referred to is confirmed; but further, the experiments show that by longer incubation of such mixtures (for 3 hours) dephosphorylation soon sets in and to a very

appreciable extent in this time. The nature of the phosphoric esters formed in the phosphorylating stage were not determined, but the work of Kalckar (7) identified them to be largely a mixture of fructose-diphosphoric and -phosphoglyceric acids. In the first hour of Experiments 1 and 2, approximately 12.5 mg. of glucose were phosphorylated by 2.08 g.

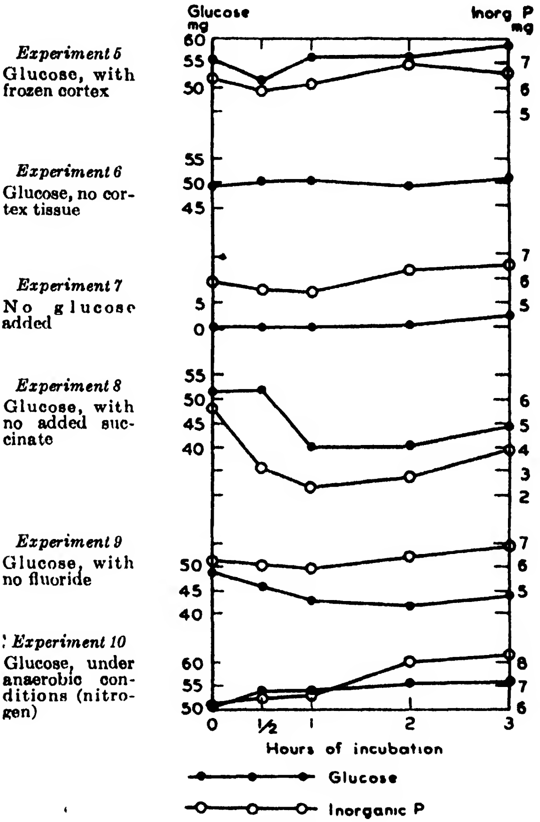

FIG. 1B

of cortex, and an average of 5.2 mg. of inorganic P disappeared. At the peak of phosphorylation in these two experiments only 0.25 mg. of the 5.65 mg. of inorganic P remained in the first experiment, and only 0.72 mg. of the 6.11 mg. in the second. Since we did not follow the nature of the P esters remaining after considerable dephosphorylation had taken place, it is only to be noted how much glucose and inorganic P remained at the end of the experiments.

It is evident from the experiments that 0.02 *M* NaF only partly inhibits kidney phosphatase. Colowick, Welch, and Cori reported that fluoride does not inhibit phosphatase, while Kalckar found the opposite. It is probably a matter of fluoride concentration although the concentrations used were not far different. In Experiment 9 where no fluoride was added but where the conditions were otherwise the same as in Experiments 1 and 2, dephosphorylation kept pace with phosphorylation. The rôle of fluoride in preventing the breakdown of phosphoglyceric acid by way of phosphopyruvic acid seems to be evident in these experiments.

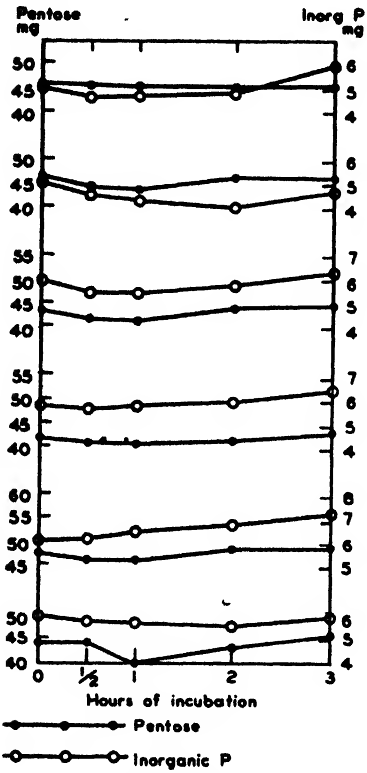

FIG. 2. Changes in Concentrations of Pentoses and Inorganic Phosphate on Incubation with Fresh Rabbit Kidney Cortex.

It is to be noted that in Experiment 9, 5.5 mg. of glucose disappeared and was not represented in any phosphorylated compound. It might have been converted into lactic acid and remained as such, or oxidation might have been carried beyond the pyruvic acid stage; that it was not oxidized without being phosphorylated is shown by the results of several of the experiments but especially by Experiments 3 and 6 where the enzymes either had been destroyed or were not present at the beginning.

Experiment 5 illustrates results such as we always found when frozen kidney cortex was used; it seems evident that denaturation of the enzymes takes place rather readily.

Experiment 6 indicates: That the analytical method used for carbohydrate determination was sufficiently accurate.

That the liberation of small amounts of some reducing substance and of some inorganic P from the cortex itself during incubation of the mixture is evident in some of the experiments, particularly Experiment 7.

Experiment 8 shows that added succinate was not required in these experiments; sufficient amounts of dehydrogenase substrates must have

been present in the cortex. This was thought probable from the beginning, since the extract had not been dialyzed. For the same reason no cozymase had been added.

It was expected in Experiment 9, where no fluoride was added, that no phosphorylation would be evident because dephosphorylation could readily take place. This was found to be the case. That considerable phosphatase was present in all of these extracts was not doubted even though the cortex has been found to have much less of this enzyme than the medullary substance.

All of the six experiments on the pentoses reported here, indicated in Fig. 2, show definitely that no phosphorylation took place with these carbohydrates. The determination of this was the main object of this research. Such non-phosphorylation of the three most common pentoses is evidence against the view that there is resorption of pentoses by the kidney tubule by means of phosphorylation.

Summary

Aerobic phosphorylation of glucose by extracts of rabbit kidney cortex is confirmed. It does not take place anaerobically.

Under the same aerobic conditions the pentoses: *l*-xylose, *d*-ribose, and *l*-arabinose are not phosphorylated. This is evidence against resorption of pentoses through the kidney tubules by phosphorylation.

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The Effect of Dry Grinding on the Properties of Proteins*

5. Keratins

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A. Wool

Native keratins are attacked by the enzymes at a very slow rate. Michaelis and Goddard (1) have shown that wool becomes more soluble and digestible if first treated with certain reducing agents. The reduction occurs at the disulfide bond of cystine, splitting them, and producing smaller fragments.

Routh and Lewis (2) compared the rates of digestion of keratin and ground wool. They found that wool reduced by thioglycolic acid (kerateine) was digested by both trypsin and pepsin at a greater rate than the ground wool. The ground wool was shown to contain appreciable amounts of water-soluble protein, a portion of which was dialyzable. These authors explained the presence of water-soluble protein in the powdered wool "by mechanical action which made the soluble material accessible to the solvent." This view alone cannot explain the observed effects on the keratins, reported here, and the other proteins studied as previously reported (3, 4, 5, 6).

Experimental

Defatted sheep wool was ground in an 8 liter ball mill for 72 hours. The wool ground for 72 hours was partly soluble in water, only 12% of the total protein dissolving. 11% of the ground wool dissolved in 0.12 *N* HCl and 21.3% dissolved at pH 8.6.

Thioglycolic acid wool was prepared by the method described by Michaelis and Goddard.

* Aided by a grant from Committee on Scientific Research of the American Medical Association, Grant No. 599.

Tryptophan determinations by the May and Rose (7) method showed that none of the fractions contained this amino acid.

A comparison of the rates of digestion by trypsin and pepsin of the ground and thioglycolic acid reduced wool has been carried out. For the pepsin experiment the proteins were suspended in a 0.12 N HCl and for the tryptic digests, a boric acid buffer at pH 8.6 was used. The concentration of protein in each case was 20 mg./cc. The rate of digestion was determined by the increase in soluble nitrogen determined by the Kjeldahl method (Fig 1).

Results

Keratin, like other water-insoluble proteins, yields water-soluble fractions after being dry ground in the ball mill. The water-soluble proteins have a characteristic odor which is acquired by all proteins after grinding. As neither the water-soluble fractions nor the water-insoluble residue remaining after grinding contained tryptophan, it would appear that this amino acid was destroyed by the grinding process.

The findings of Routh and Lewis have been corroborated. Fig. 1 shows that kerateine is digested more rapidly than ground wool by both trypsin and pepsin.

The ground wool gives a direct nitroprusside test indicating the splitting of disulfide bonds. This test is given by all of the sulfur-containing proteins thus far ground. However, the importance of the breaking of this linkage in producing water-soluble protein cannot be too great. The fact that ground wool is digested at a slower rate than the reduced wool would point to the possibility that other linkages were being broken in grinding. Other linkages, such as hydrogen bonds and a few peptide linkages, if broken would result in fragments being produced having greater solubility. Polypeptide chains may be split from the polypeptide grid. Since proteins having a low sulfur content (gelatin, casein, elastin) also yield water-soluble proteins, thus undergoing fragmentation, it would seem that the disulfide bond is only partially responsible in the high sulfur containing proteins and only of minor importance in the low sulfur proteins for the breakdown observed.

B. Ox-Horn Keratin

20 grams of ox-horn keratin were obtained by shaving the horn of an ox and placed in a large ball mill and ground for 48 hours. After this time the powder was extracted and the grinding repeated until four such fractions, soluble in water, were obtained. These fractions have been analyzed for their nitrogen, sulfur, tyrosine, and tryptophan content. The amount of nitrogen diffusible through Visking casing shells was determined (Table I).

To determine the digestibility of the water-soluble proteins by pepsin and trypsin, the fractions were dissolved so that the protein concentration was 20 mg./cc. The solutions for peptic digestion were made up in 0.63% HCl and for

TABLE I

Analysis of the Water-Soluble Proteins of Ox Horn Keratin

Protein	Nitrogen *per cent*	Sulfur *per cent*	Tyrosine *per cent*	Tryptophan *per cent*	Dialyzable *per cent*
Native Keratin*.....	15.73		4.80	1.8	
Keratin 1st gr...... ..	15.32	4.16	3.41	0	74.3
" 2nd gr..	15.13	4.32	3.54	0	76.4
" 3rd gr. ...	14.97	4.02	3.67	0	80.3
" 4th gr......	14.51	4.36	4.14	0	72.7
" Residue*....	13.02		3.31	0.67	

* Values corrected for the ash content.

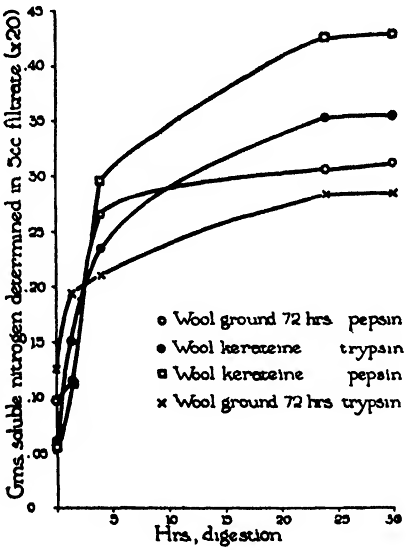

FIG. 1

Comparison of the Digestion of Ground Wool and Wool Keratin by Trypsin and Pepsin

the tryptic experiment in a buffer at pH 8.2. The rate of hydrolysis was followed by the Sorensen titration. The pepsin concentration was 10 mg. in each cc. (1–3,000 pepsin). The trypsin (Fairchild) was added so that the ratio of substrate to enzyme was 4–1 (Figs. 2 and 3).

Results

As shown with some of the other proteins studied, the nitrogen content decreases in successive water-soluble fractions. They contain no tryptophan, since no color developed when the May and Rose method was used. Tyrosine is fairly constant in the first three fractions, that of the fourth being somewhat higher. All fractions contain over 4% sulfur although the state of the sulfur, whether organic, inorganic or both, remains undetermined.

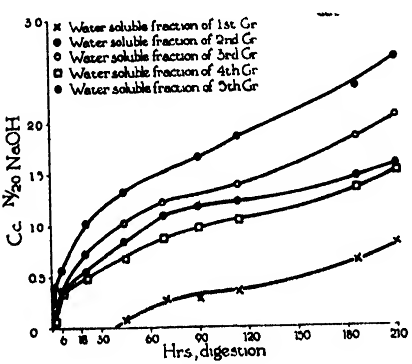

FIG. 2

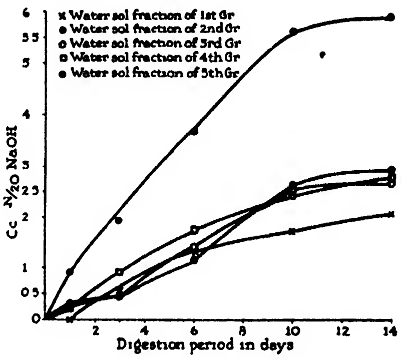

FIG. 3

FIG. 2.—Comparison of Rates of Digestion of Water-Soluble Proteins from Ox Horn Keratin by Pepsin.

Formal titration using $N/20$ HaOH.

FIG. 3.—Comparison of Rates of Digestion of Water-Soluble Proteins from Ox Horn Keratin by Trypsin.

Formal titrations using $N/20$ NaOH.

A comparison of the rates of digestion by trypsin and pepsin indicates that the second water-soluble fraction is digested at a more rapid rate than the others. According to Routh, the water-soluble protein split from wool is not attacked by trypsin or pepsin. This points to the possibility of there being a distinct difference in the structure of wool and ox-horn keratin.

The absence of tryptophan in the water-soluble proteins and the low concentration of this amino acid in the water-insoluble residue would seem to indicate a marked destruction of tryptophan in the grinding process.

Some protein may be extracted from the ground ox-horn keratin by 95% alcohol. This protein is soluble in alcohol-ether mixture, is precipitated from solution by the addition of water and gives the Biuret reaction.

The water-soluble fractions give a violet biuret test. They are precipitated by phosphotungstic acid, picric acid, by half-saturation with ammonium sulfate, and yield only slight precipitates with trichloroacetic acid. They are non-coagulable by heat and are not precipitated by acid or alkali.

Conclusions

We have pointed out that the dry grinding of various types of proteins brings about wholesale degradative changes. Similar changes have been shown to take place in wool and ox-horn keratin. The production of water-soluble protein is explained more satisfactorily by the above facts, rather than by a viewpoint held by other workers that the process is a purely physical effect.

The fibrous proteins are believed to have a grid structure consisting of polypeptide chains. The importance of lateral links between parallel polypeptide chains, the bond of the disulfide group of cystine and the polar link of diamino and dicarboxylic acids in determining the stability of keratin has been shown by Speakman (8). The water-soluble proteins are formed perhaps because of the breaking of these bonds as well as others in the grinding process.

Kerateine is formed by reducing agents due to conversion of disulfide. to sulfide. Since thioglycolic wool is different from wool which has been ground, it would seem that other linkages were also attacked in dry grinding which are unaffected by reduction. The use of this method may prove a valuable aid in determining structural details in keratin as well as of other proteins.

Summary

1. Ox-horn keratin, like wool, yields water-soluble protein upon grinding.

2. The water-soluble fractions contain no tryptophan.

3. The nitrogen content decreases with successive grindings, a fact noted with some of the other proteins studied.

4. The water-soluble proteins contain high percentages of dialyzable material. This indicates that the fragments are of small molecular size.

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The Effect of Dry Grinding on the Properties of Proteins*

6. Peptone-Proteose

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Introduction

It has been shown by us (1, 2, 3, 4) that the protein molecule can be broken down into smaller, water-soluble molecular components by dry grinding.

We have reported that ground coagulated ovalbumin yields water-soluble fractions which are heterogeneous as shown by electrophoresis and diffusion data. The latter data indicates that the smallest particles have molecular weights of 10,000–12,000.

The results of the analyses reported here are intended to show that the proteose fraction of proteose-peptone mixture is further split or converted into peptone.

Experimental

Fifty grams of Witte Peptone, which contained 14.72% of the total nitrogen as proteoses determined by precipitation with trichloroacetic acid, were ground in a ball mill in a manner previously described (1). Samples of the ground peptone were removed at intervals of time and the amount of nitrogen precipitable by trichloroacetic acid from solutions of these was determined. The change in this value was taken to indicate the change from proteose to peptone.

Tryptophan, tyrosine, total nitrogen, and ash were also determined. Tryptophan was determined by the May and Rose method (5). The content of tryptophan in the casein control was taken as 2.2%. The tyrosine values were obtained from analysis of the fraction by the Folin and Marenzi method (6). Nitrogen determinations were carried out by the macro-Kjeldahl method (Table I).

All samples were brought to constant weight in an electric oven at 70°C. and then kept in a desiccator over sulfuric acid until analyzed.

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* Aided by a grant from Committee on Scientific Research of the American Medical Association, Grant No. 599.

Results

Even after short periods of grinding, the powder was more readily dissolved in water. During a period of 200 hours of grinding 66.23% of the proteose nitrogen was converted into peptone nitrogen.

In the paper on coagulated hemoglobin (4) it was pointed out that there was a decrease in the total nitrogen of successive water-soluble fractions. The total nitrogen of the proteose-peptone decreases only after prolonged grinding. During the first 88 hours of grinding there is only a slight decrease in the total nitrogen; after 250 hours' grinding the nitrogen content has decreased from 15.50, at the beginning of grinding, to 12.58%.

TABLE I

*Peptone Experiment**

Hours ground	Nitrogen in orig. sol.	Nitrogen not ppt'd. by CCl_3COOH	Nitrogen ppt'd. by CCl_3COOH	Total N_2	Tryptophan	Tyrosine	Ash Content	Proteose
	g.	*g.*	*g.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	0.01854	0.01581	0.00273	15.50	4.91	5.02	0.68	14.72
17	0.01960	0.01704	0.00256	15.48	4.88	5.01	1.37	13.06
24	0.0212	0.01884	0.00236	15.40	4.69	5.09	1.99	11.13
64	0.01578	0.01414	0.00164	15.40	4.30	4.92	2.00	10.39
88	0.01697	0.01593	0.00104	15.36	4.46	4.91	2.06	6.12
128				14.95	4.06	4.98	2.09	
152				14.84	4.09	4.73	2.24	
176	0.01715	0.016170	0.00098	14.72	4.08	4.20	2.50	5.13
200	0.02007	0.01907	0.00100	14.11	3.94	4.13	2.56	4.97
250				13.55	3.97	4.12	2.62	

* All values in the above table are corrected for the ash content.

That tryptophan is decomposed by grinding has been demonstrated previously. The table shows that grinding of peptone over a period of 250 hours causes a decrease in the tryptophan content by 19.14%.

Tyrosine is not decomposed as easily as tryptophan since grinding proteose-peptone over a period of 152 hours produced practically no change. In later periods of grinding there was a steady decomposition of the tyrosine so that 17.90% was changed at the end of 250 hours.

The ash content steadily increases but much less than might be expected. This may be due to the hygroscopic nature of peptone so that, as the mill is opened to remove the samples, enough moisture is absorbed so that the peptone forms a cushion about the balls and does not allow them to break as easily as they otherwise would.

Discussion

The composition of proteose-peptone is exceedingly vague. Variations in the proteose content of different preparations is great so that it will not be possible to reproduce exactly the data stated in this paper on other preparations. Armour's peptone contained no proteose and is completely peptone. Different samples of Witte peptone contain varying amounts of proteose.

It seems quite definite that proteose is converted into lower protein degradation products. How this is brought about cannot be stated. It may be assumed that some peptide splitting occurs but other bonds are probably more important. We have obtained by grinding coagulated ovalbumin water-soluble fractions which were electrophoretically heterogeneous and by diffusion measurements were of small molecular size. The same forces which break down the larger protein molecules must be operative in breaking down the smaller components into simpler molecular form.

The steady loss of nitrogen must be through deaminization. Routh has shown that oxidative changes occur during grinding. It is quite possible that oxidative deaminations of the free amino group also takes place and that this may account for the decrease in total nitrogen on prolonged grinding. The amide nitrogen may also be split off and may account for the loss in total nitrogen.

Summary

1. Grinding peptone in the ball mill results in a decrease in the nitrogen precipitable by trichloroacetic acid. This indicates that there is a conversion of proteoses into peptone by the grinding process.

2. There is a definite decrease in the nitrogen (%) as the grinding proceeds which may be interpreted as indicating that there is deaminization of amino or amide groups.

3. The tryptophan content decreases indicating a destruction of this amino acid by the grinding process.

4. Tyrosine is destroyed less readily than tryptophan, very little change occurring during the first 152 hours of grinding in the ball mill.

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Steroids and the Specificity of the Pettenkofer Reaction

I. Qualitative Studies[1]

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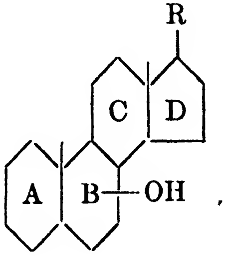
Received December 18, 1943

Introduction

The Pettenkofer reaction (1) has been modified by Schmidt (2) for use in the quantitative determination of cholic acid. In this paper Schmidt's procedure has been utilized in a qualitative test. It was our purpose to determine qualitatively the specificity of the Pettenkofer reaction as applied to steroids.

In the past it has been generally conceded that a hydroxyl in the steroid Ring B was necessary for the Pettenkofer reaction (5). This



assumption has taken into account neither such compounds as apocholic acid nor the position of the hydroxyl when it occurs in Ring B. In our investigation apocholic acid and other cholenic acids produced strong positive tests, while hyodesoxycholic acid with one of its hydroxyl groups in Ring B gave a negative result. Certain compounds earlier considered to produce a positive test did not do so when carefully

[1] This paper was presented before the Chemistry Section of the Missouri Academy of Science Meeting held in Kansas City, Mo., April 16–18, 1942.

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purified. Thus, in 1911, H. Fischer (3) stated that lithocholic acid gave a positive Pettenkofèr reaction. A sample of lithocholic acid[3] was prepared (4) and crystallized from dilute methyl alcohol, dilute acetic acid and finally from acetone. The Schmidt modification of the Pettenkofer reaction gave negative results. Desoxycholic acid[3], purified according to known methods, was converted to methyl 3,12-dibenzoxycholanate and recrystallized several times from S.D. 3A alcohol. This derivative was then saponified on refluxing five hours with 2 N sodium hydroxide solution. The desoxycholic acid precipitated from this reaction was isolated and dried. It was recrystallized three times from glacial acetic acid. The acetic acid-choleic acid produced a negative Pettenkofer test in the modified procedure. Desoxycholic acid was obtained from the choleic acid complex by the usual method, and a negative Pettenkofer test also resulted with it.

The compounds that gave a positive test may be arranged into general classes, 1. those saturated compounds that possess a hydroxyl group at C_7, and 2. compounds that possess a double bond in one of the rings of nucleus. Dehydration may take place easily in steroids with a hydroxyl at C_7, and apocholic acid is characteristic of the dehydration product of cholic acid. Unsaturated carboxylic acids gave well defined positive tests. Of especial interest is the fact that dehydro-trans-androsterone acetate, an unsaturated steroid, gave a very strong reaction and this may be useful in detecting this compound or the deacylated material in hormone mixtures[4]. A keto-grouping at C_7 in the steroids which were saturated gave negative Pettenkofer reactions. α,β-Unsaturated ketones gave questionable reactions. It is reasonable to assume, that steroid carboxylic acids unsaturated in one of the four rings will give a positive Pettenkofer test.

Experimental

The steroids, substituted in various positions, were tested according to a standardized technic. A sample of 5 mg. was dissolved in 2.5 cc. of 60% acetic acid solution in a test tube and heated to 70° C. One cc. of 0.9% furfural solution was added to the dissolved sample and the test tube placed in a bath of water at 74° C. and allowed to remain two minutes when 6 cc. of 16 N sulfuric acid were added. The

[3] We gratefully acknowledge the assistance of J. Linsk for the preparation of purified lithocholic and desoxycholic acids.

[4] Dr. T. F. Gallagaher has indicated this application to us in a personal communication.

contents of the tube were then gently mixed and heated 10 minutes and 7 cc. of 60% acetic acid added. A positive reaction was one in which a blue color developed although with certain compounds this color was nearly violet or purple. The conditions of the test were slightly more strenuous than the ordinary quantitative procedure (2). Compounds difficultly soluble in 60% acetic acid were first dissolved in a minimum volume of ethyl alcohol before the 2.5 cc. of 60% acetic acid was added.

Results of the Modified Pettenkofer Reaction

Negative Reaction

3-Acetoxy-*bis*-*nor*-cholanyldiphenylethylene
21-Benzal-12-hydroxypregnanol-3-one-20
Cholesterol (?)
17-Carbethoxyestradiol (Green)
Dehydrocholic acid
Dehydrodesoxycholic acid
Desoxycholic acid
Desoxycholic acid obtained from reduction of 3,12-dihydroxy-7-ketocholanic acid
3,11-Dihydroxy-*allo*-*etio*-cholanic acid
3,12-Dihydroxy-7-keto-cholanic acid
Estradiol
Estrone
3-Hydroxy-12-keto-*nor*-cholanic acid
12-Hydroxypregnanol-3-one-20
Hyodesoxycholic acid
3-Keto-cholanic acid
3-Keto-11-hydroxy-*allo*-*etio*-cholanic acid
Lithocholic acid
bis-*nor*-Lithocholic acid
Methyl 3-benzoxy-11-bromo-12-keto-*bis*-*nor*-cholanate
Methyl 3-benzoxy-7-keto-12-hydroxycholanate
Methyl 3-benzoxy-7,12-diketocholanate
Methyl 3,6-diketo-Δ^4-cholenate (Straw color)
Methyl 3-hydroxy-6-ketocholanate
Methyl *nor*-lithocholate
Methyl reductodehydrocholate
Progesterone
Reductodehydrocholic acid
Stigmasterol
Stigmasterol acetate
Stigmasterol acetate tetrabromide

Positive Reaction

Apocholic acid
Chenodesoxycholic acid
Cholic acid

nor-Cholic acid
bis-nor-Cholic acid
Dehydro-*trans*-androsterone acetate
3-Hydroxy-Δ^5-cholenic acid
12-Hydroxy-Δ^8-cholenic acid
Methyl 12-hydroxy-Δ^8-cholenate
3-Succinic acid ester derivative of methyl cholate
Triformyl cholic acid
3,7,12-Trihydroxy-*nor*-cholanyldiphenylcarbinol

Summary

The Schmidt modification of the Pettenkofer reaction was applied to a number of steroids. All steroids that possessed at C_7 a hydroxyl or a group easily changed to a hydroxyl group gave a positive test. Δ^3, Δ^5, and Δ^8-Monocholenic acids and their esters gave positive results. The α,β-unsaturated ketone grouping did not produce the characteristic color of the Pettenkofer reaction. Dehydro-*trans*-androsterone acetate gave a positive reaction which should be useful in the detection of this compound.

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Serum Albumin as a Protective Colloid for Euglobulin in the Formol Gel Reaction

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Introduction

The reaction between proteins, especially blood proteins, and formaldehyde has been studied from two entirely different aspects: (1) Addition of 5% (neutralized) CH_2O to normal serum prevents the serum proteins from coagulation by heat through formation of the so called methylene proteins. (2) Addition of a few drops of a strong CH_2O solution to plasma and to certain pathological sera produces opalescence and, after a certain interval, complete clotting. The latter reaction known in pathological cases as the formol gel reaction (FGR), occurs regularly with normal *plasma* of all kinds and is due to the presence of fibrinogen. We have found that a 1% solution of purified fibrinogen clots almost instantly on the addition of 2 to 3 drops of a neutral 40% formaldehyde solution. With *serum* the reaction occurs under pathological conditions only (hyperglobulinemia), and therefore, has been applied for diagnostic purposes.

We have investigated the quantitative conditions which lead to a positive FGR by combining the isolated proteins in physiological saline solutions in varying amounts but with the temperature and pH kept constant.

Most authors, including Bing (1), deVries (2), and Napier (3), believe that the *albumin* plays no intrinsic part in the formol gel reaction (FGR). In our studies we found that the albumin, a serum protein of a relatively low molecular weight (70,000), interacts with the euglobulin (mol. wt. 160,000) as a typical "protective colloid."

Methods

I. The Formol Gel Reaction

The reaction (4, 5) is obtained by the addition of 2 drops of a 40% formaldehyde solution to 1 cc. of the protein solution in a test-tube of 8 mm. bore, with shaking. The mixture is allowed to stand at a temperature of about 20°C. Both the protein and the formaldehyde solutions are brought to pH 7.4 to 7.6 with $NaHCO_3$-Na_2CO_3. The results are indicated at intervals as follows: +, gel; ++, soft clot; +++, firm (opalescent clot); ++++, instantaneous clotting.

II. Preparation of Serum Protein Fractions

The total *globulin fraction* of the serum protein was obtained by precipitation of the serum with ammonium sulfate at one-half saturation (we applied the prescription given by Reye (6)). The precipitate was dissolved in water and dialyzed. When complete removal of the salt had occurred, the euglobulin that precipitated out was centrifuged off, washed with distilled water, and dissolved to a concentrated solution by adding the necessary amount of a 10% sodium chloride solution. Sodium carbonate was added to bring the pH to 7.4. The globulin-free filtrate was acidified with a half saturated solution of 0.1 *N* H_2SO_4 and $(NH_4)_2SO_4$, and the precipitated albumin filtered off, redissolved in distilled water, completely dialyzed, and brought to dryness in a high vacuum at room temperature. The pseudoglobulin fraction, being the water-soluble part of the total globulin fraction, was prepared from the euglobulin filtrate by reprecipitation, filtration, dialysis, and desiccation in a high vacuum.

For our experiments, the albumin, pseudoglobulin, and euglobulin fractions were prepared from sheep serum; some preparations have been obtained from cattle serum and from human serum. The biological specificity of the various proteins does not interfere with the reaction.

For the experiments concerning the interaction between euglobulin and pseudoglobulin with albumin we used a 5% albumin (saline) solution as a standard.

Tables I to III represent the results with the FGR as applied to various protein solutions.

Discussion

Table I indicates that a 5% albumin solution will neither gel nor clot on the addition of formaldehyde even after 90 hours. Pseudoglobulin, requiring 16 to 24 hours to clot in solutions of 15 and 10% respectively, will clot in 6 hours upon addition of 5% albumin. A 5% pseudoglobulin solution which takes 90 hours to clot, will clot in 20 hours on the addition of a 5% albumin solution. This acceleration can be explained as a simple addition phenomenon. The effect of albumin on the euglobulin FGR is entirely different: relatively small concentrations of albumin tend to inhibit the reaction. A very strongly positive reaction which occurs in 2 minutes with a 4.1% solution of euglobulin in saline solution (Table II)

TABLE I

Formol Reactions of Serum Albumin (A) and Serum Pseudoglobulin (P)

Time	Concentration of saline solution									
	20%		15%		13%		10%		5%	
	A	*P*	*A*	*P*	*A*	*P*	*A*	*P*	*A*	*P*
hrs.										
2	0	++		0	0	0	0	0	0	0
16	0			++	0	0	0	0	0	0
24	++				++	++	0	++	0	0
48							++		0	0
90									0	++

TABLE II

Formol Reaction of Serum Euglobulin

Time	Concentration of saline solution					
	6.2%	4 1%	3.1%	2.5%	2.07%	1.5%
1 min.	+++	0	0	0	0	0
2 "		+++	0	0	0	0
45 "			+++	0	0	0
5 hrs.				++	0	0
8 "					++	0
22 "					+++	+

TABLE III

Formol Reaction of Euglobulin in 5% Albumin (Saline) Solution

Total protein. Euglobulin.	8 7% 6 2%	7.3% 4 1%	6.8% 3 1%	6.0% 2.07%
1 min.	+++	0	0	0
5 "		0	0	0
45 "		++	0	0
60 "		+++	0	0
5 hrs.			+	0
6 "			++	0
8 "				0
24 "				0

takes 60 minutes if 3.2% albumin is added (Table III); in a dilution of 3.1% the FGR of euglobulin is retarded from 45 minutes to 5 to 6 hours by the addition of 3.7% albumin. This retardation phenomenon, the protective property of the albumin, seems to have a limit; in the 6.2% dilution of euglobulin, the addition of 2.5% albumin had no retarding effect.

As noted in Table II the FGR of euglobulin alone is strong when in relatively small concentrations as compared with pseudoglobulin and especially with albumin; as far as is known, fibrinogen is the only protein which parallels euglobulin in this respect.

This work had its inception in the attempt to denature beef plasma or serum with formaldehyde for use as material for transfusion into humans. When it was found that albumin retarded the clot of euglobulin in the FGR, we applied this principle in an attempt to retard the normal clotting of fibrinogen (plasma) by increasing the albumin concentration. This, however, was found not to apply, for, when albumin was added to blood or plasma heparinized with calcium chloride, the clotting was accelerated. Further investigation is required to prove the significance of the relation between euglobulin and albumin; that the serum albumin fraction has an influence on the stability of the euglobulin has been made plausible under other conditions by experiments on protein coagulation in drops (Boehm (7)) as well as on comparative fractionation of the serum proteins with Na_2SO_4 and with electrophoresis (Taylor and Keys (8)).

Conclusion

The addition of serum albumin retards the formol gel reaction of the serum euglobulin in various concentrations.

Our thanks are due to Dr. Joseph Guedemann who gave us his kind assistance and has made available for us the facilities of the clinical laboratory of Beth David Hospital.

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The Regular Urinary Excretion of a Chromogenic Substance during Pregnancy

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Introduction

For an early diagnosis of pregnancy the biological assay method introduced by Aschheim and Zondek (1, 2) and modified by Friedman (3) is in general use. This test is based on the presence of chorionic gonadotropin in pregnancy urine.

Biological assay methods are time consuming and expensive and therefore various attempts have been made to find a simple *chemical* pregnancy test. In a recent patent Gutschmidt and Gutschmidt (4) claimed that the addition of iodine or of iodine liberating reagent mixtures to heated pregnancy urines, would produce a red pigment. The simplicity of this pregnancy test attracted our attention, and this report deals with an investigation of the claims of the inventors and with observations on the nature of the pigment and of the chromogenic material.

Experimental

Optimal Testing Conditions. According to the patent, a solution of iodine in potassium iodide solution can be used as reagent. The solution used throughout this investigation contained 1.5 g. iodine and 2.5 g. KI in 100 ml. water. The inventors mention that the red pigment can be extracted with amylalcohol and can then be seen better. The lack of further details in the patent made it necessary to work out optimal conditions for the test. The following procedure was finally adopted: The pH of the urine to be tested was brought to approximately 5.5 using nitrazine paper as indicator. Urine samples of 5 ml. each were pipetted into two test tubes and heated to 70–80°C. When this temperature was reached, 0.3 ml. reagent were added to one tube, 0.6 ml. reagent to the other tube. The material was mixed for about 20 seconds and 2 ml. amylalcohol were then added to each tube. After thorough mixing the samples were centrifuged for a few minutes. In the case of a positive test, the clear amylalcohol layer had a pink to red color

and showed two narrow absorption bands at 560 and 525 mμ. The pigment is unstable, especially in water solution Prompt addition of amylalcohol after the pigment had been formed was therefore important.

Results with Normal and Pregnancy Urines. The test in its final form was positive in 163 (97%) of 168 different pregnancy urines. There was no relation between month of pregnancy and amount of pigment obtainable. In order to be useful as a pregnancy test, the outlined procedure should not give positive results with urines from non-pregnant persons. While the patent stated that normal urines would remain yellow or assume the color of the reagent, it seemed advisable to perform the test with a number of normal urine samples. The results were as follows:

Male donors, 40 out of 65 urines (62%) gave a positive test.

Non-pregnant female donors, 46 out of 77 urines (60%) were positive. Most pregnancy urines contained more of the chromogenic substance than positive reacting normal urines, but overlapping in color intensity did occur in about 20% of the samples tested. Such large possibilities for error render this patented test useless for the diagnosis of pregnancy. Despite this it seemed worthwhile to investigate the nature of the chromogen and the red pigment, as the result of such an investigation might eventually further the understanding of metabolic changes during pregnancy.

Comparison with Histidinuria. In some aspects, the chromogenic material under discussion shows similarities to histidine.

The reagent used to determine histidine colorimetrically is bromine, which is a halogen and an oxidizing agent as is iodine. Furthermore, histidinuria once was considered to be specific for pregnancy (5–8). It was found later, however, that the amounts of histidine in normal urines fluctuated considerably from day to day and from person to person (9–11) and that large quantities of this amino acid were not only present in pregnancy urines but occasionally in normal urines from both sexes and frequently in urines from patients suffering from a variety of diseases (12). Langley concluded from distribution curves of histidine excretion in normal and pregnancy urines (13), that due to overlapping of the curves, about 16% of the pregnancy cases could not be diagnosed as such with certainty. The determination of histidine, for the reasons enumerated, cannot be used as a chemical pregnancy test.

Neither histidine in water solution nor histidine added to urines which before had given a negative test with the iodine reagent, formed a red

pigment under the conditions of the test. The chromogen producing the red pigment is therefore not identical with histidine.

Properties of the Chromogen. The chromogen could not be extracted from neutral, acid, or alkaline urine samples with ether, chloroform, n-butylalcohol, isoamylalcohol, and benzene. It was not volatile with water vapors and not damaged on evaporating urine samples to dryness on the water bath. Extracts made from dry residues with ether, methylalcohol, ethylalcohol, acetone and chloroform were free of chromogen.

While boiling at pH 5.5 did not destroy the chromogen the optimal color intensity with the iodine reagent was only obtained when the samples were cooled to 70–80°C. before the reagent was added. Boiling in 2 *N* HCl for one hour had little effect on the outcome of the test. However, when the heating was continued for two hours, the intensity of color produced with previously strongly positive urines became considerably weaker. After acid hydrolysis for one hour, the chromogen could not be extracted with ether or benzene from the acid (or alkalinized) solution. This excludes among other compounds, estrone or any ether soluble phenol coupled with glucuronic or sulfuric acid as being the chromogen. The chromogen was not very stable in alkaline solution. If urines giving a strong positive test were stored at 4°C. for longer periods, the test remained only positive if the pH of the sample was 7 or less. A pH of 7.5 or 8 was sufficient to lead to the disappearance of the chromogenic material within several weeks in the cold, while acid samples showed the color reaction with apparently undiminished strength even after 3–4 months.

The chromogen dialyzed quickly through cellophane membranes. Proteins and urochrome (14) remained in the dialyzing bag.

In contrast to chorionic gonadotropin (15) the chromogen was not adsorbed on benzoic acid. Water solutions of chorionic gonadotropin (100,000 I.U./liter) or their mixtures with negative reacting urines, did not form a red pigment on addition of the iodine reagent.

The chromogen passed unadsorbed through a column of aluminum hydroxide, but was quantitatively removed from urine samples on treatment with decolorizing charcoal, using 1 g. for 20 ml. pregnancy urine. Attempts to elute the chromogen from the charcoal with various solvents, such as HCl, NH_4OH, CH_3COOH, and phosphate buffers, were unsuccessful. Histidine behaves similarly (9). Previous treatment of Pfannstiehl charcoal with H_2S did not change the activity enough to make an

elution possible. When only 20 g. charcoal were used for 10 ml. urine, the filtrate was pale yellow and gave the iodine test as strongly as the original urine. This excludes urothion (16) from being the chromogen, as it is quantitatively adsorbed under these conditions.

The chromogen was not precipitated by acetone, barium hydroxide, silver nitrate, lead acetate, phosphotungstic acid, or ammonium sulfate. Increased salt concentrations (for example 0.4 saturation with ammonium sulfate) have however an inhibitory effect on the pigment formation with iodine.

Properties of the Pigment. The pigment could be extracted from its water solution with amylalcohol and iso-butylalcohol. It was insoluble in ether, chloroform, benzene, toluene, and petrolether. The pigment was more stable in amylalcohol solution than in water. But here the color faded also gradually, and after standing at room temperature for several hours, all red pigment had disappeared. Renewed treatment of such faded solutions with iodine did not reverse this change. Toward acids and bases the pigment behaved like an indicator, being red in presence of acids and yellow in alkaline solution. The change from the red acid to the yellow salt was reversible and apparently not accompanied by any destruction of the pigment, provided that the pH was kept between approximately 5 and 8.

Absorption curves of the pigment dissolved in amylalcohol were obtained with a Cenco-Sheard spectrophotelometer in connection with a Leeds and Northrup galvanometer # 2500-g. Type R. Fig. 1 shows the absorption curve of the pigment, as it was obtained when the urine sample had been adjusted to pH 5.3 (glass electrode) before addition of the reagent. Fig. 2 shows the absorption curves obtained if, before addition of the reagent, the pH of the urine sample had been brought to 6.4 (Curve A) and to 7.4 (Curve B). The positions of the maxima were as follows:

Fig. 1	(pH 5.3)	494, 525 and 560 mμ.
Fig. 2	(pH 6.4)	523 and 561 mμ.
	(pH 7.4)	475, 492 and 523 mμ.

Comparison with Red Urinary Pigments and Their Chromogens

The chromogen and pigment under discussion were not identical with any of the urinary pigments and chromogens listed below.

Pigment or chromogen	Main distinguishing characteristic
Urobilinogen	Solubility in ether
Coproporphyrin I	Solubility in ether
Urobilin	Solubility in ether after reduction

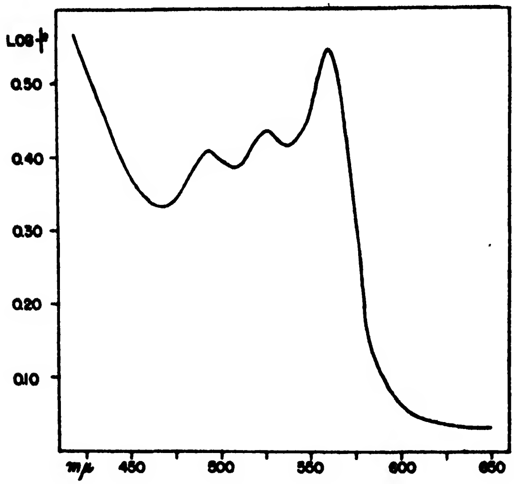

FIG. 1

Absorption Curve of the Amylalcohol Solution of the Pigment, Produced by the Reaction of Pregnancy Urine (pH 5.3) with Iodine

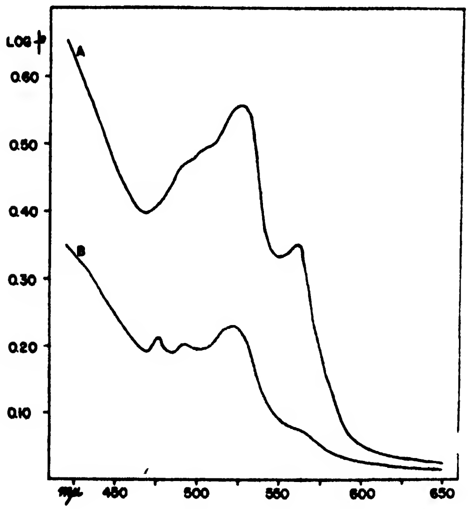

FIG. 2

Absorption Curve of the Amylalcohol Solution of the Pigment, Produced by the Reaction of Pregnancy Urine with Iodine

Curve A: pH of urine before addition of the reagent was 6.4.

Curve B: pH of the sample before addition of the reagent was 7.4.

Uroporphyrin I and III	Adsorption on aluminum hydroxide
Bilirubin	Precipitation with Calcium chloride
Indigored	Solubility in ether
Uroerythrine	Removal with amylalcohol without effect on outcome of test
Urocarmin	No characteristic absorption bands
Scatolered	Different absorption spectrum
Indolignone pigments	Solubility in chloroform
Urorosein*	Different absorption spectrum
Indole acetic acid	No pigment formation with iodine

In a number of urines the intensity of the indican test paralleled the intensity of the iodine test, i.e. urines containing only traces of our chromogen formed also very little indigoblue on addition of Obermayers reagent (20 mg. $FeCl_3$ in 100 ml. conc. HCl), while urines rich in our chromogen yielded large amounts of indigo.

The following procedure made it possible to separate the indigo forming chromogens (indoxyl, indican, and indoxyl glucuronic acid) from the chromogen contained in pregnancy urines:

To a mixture of 50 ml. pregnancy urine with 30 ml. water and 12 ml. HCl (1:5), were added 8 ml. 5 per cent $BaCl_2$ solution, and after one hour standing, the $BaSO_4$ precipitate was removed by filtration. The yellow filtrate, which gave a strong indican and iodine test, was boiled under reflux for 30 min. Barium sulfate and indigo formed during this hydrolysis and were removed by filtration. The filtrate gave a strong iodine test, but did not form indigo on addition of Obermayer's reagent.

It may be mentioned here, that a large variety of compounds of biochemical interest were tested with the iodine reagent in search for the pregnancy chromogen. Among the substances tested, tyrosine (in phosphate buffer solution pH 6) reduced considerable amounts of iodine but failed to form a pigment. Adrenaline, alloxan, and dioxyphenyl alanine formed red pigments under the conditions of our test. These pigments however, were not soluble in amylalcohol and showed no distinct absorption bands.

Diet and Chromogen Excretion. The occurrence of small amounts of chromogen in a considerable number of urines from normal persons,

* Urorosein showed some similarities with our pigment. It is fairly unstable, it shows the same indicator behavior as our substance and there are two maxima in the visible portion of its absorption spectrum. But these maxima are not at the same positions as those of our pigment and furthermore the absorption curve for urorosein is quite different in shape. The color of urorosein prepared from indole acetic acid is different from the color of our pigment and dark red urorosein solutions show spectroscopically much weaker and broader bands than do light red solutions of the pigment formed by the action of iodine.

made it seem possible that the diet of such persons might supply the chromogen or its precursor, especially as the occurrence and disappearance of the chromogen in successive daily samples was quite irregular. If a given diet would once produce the excretion of the chromogen, one would expect to be able to reproduce its excretion at will.

Through the courtesy of Dr. George W. Thorn, we obtained daily urine samples collected from three healthy young men, who had undergone the monotonous task of consuming an identical diet for about 11 weeks. Each day these persons obtained generous allotments of orange juice, eggs, white bread, butter, jelly, milk, beef, potatoes, lettuce, tomatoes, canned pears, raw peas, raw carrots, and celery. A total of 44 neutral or acid samples (24 hour collections) were tested and all formed the red pigment on treatment with iodine. Further dietary studies are scheduled with the intention of confirming and tracing the dietary origin of the chromogen in these cases.

Summary

1. Pregnancy urines contain a chromogen which on suitable treatment with iodine forms a red pigment.

2. The occurrence of this chromogen is not restricted to pregnancy urines. It also occurs irregularly in normal urines from men and women in small amounts.

3. The claim of the German Patent 680223 that a positive iodine test indicates pregnancy is untenable.

4. The new pigment or its precursor are not identical with any of the known red urinary pigments or their chromogens.

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Caffeine and Methionine as Choline Substitutes in Tropical Heat

Preliminary Communication

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Introduction and Experimental

Deficiency of labile-methyl supply in the diet is known to bring on fatty degeneration of the liver and acute hemorrhagic changes in the kidneys of weanling rats on about the eighth day; it has not been clear, however, whether these same methyl groups are necessary for subsequent growth and development of the animals. Growth of rats on choline-free diets (and containing no choline substitutes) is not much below optimal levels in cool environments after the eighth day danger period has passed; in tropical heat, however, subsequent growth is sharply dependent upon adequate supplies of choline, with best development being reached only when the dietary choline has been increased several times above that needed in cool environments,—5 g./kg. instead of 0.75 g. (1). It thus becomes important to determine whether other suppliers of labile-methyl can replace choline in this later support of growth under the heat loss difficulties of hot environments.

Methionine was chosen as the most likely choline substitute, and tests soon showed it to be fully capable of supporting normal rat growth in the heat. However, ordinary food sources for methionine would probably be just as deficient as would those for choline in supplying the large amount of labile-methyl needed in hot surroundings. In surveying the field of other possible substitutes for one which would be readily available in ample amounts, the thought came that perhaps caffeine might serve the needed purpose. This compound has three labile methyl groups, just as choline does, and in the body it gives up all or part of them in its conversion toward xanthine (2). It has the advantage of

large scale tropical production and almost universal use as a beverage. If it could meet the hot climate need for additional labile-methyl supply, a most fortunate solution to this phase of the tropical nutrition problem would be provided.

Weanling white rats (Sprague-Dawley males) were placed in rooms at 90–91°F. and 60–70% RH* and at 68°F. Diets used were those previously described as optimal for hot and cold room rats (1). In testing for the efficacy of choline substitutes, all choline was omitted and its place taken by the methionine or caffeine as indicated in the accompanying table of results. Weighings of the animals were made weekly and a record kept of deaths at the 8–9 day danger period. There were 12 rats to each group.

TABLE I

Methionine and Caffeine as Choline Substitutes

	At 68°F.					At 90–91°F. and 60–70 % RH				
Choline rat series as control										
Choline in diet (g./kg.)	0.0	0.4	0.75	1.5	3.0	0.0	0.4	1.5	3.0	5.0
Deaths on 8th day	11/16	0/12	0/12	0/10	0/8	9/16	0/10	0/10	0/8	0/31
Weight gain (g.) during 2nd 3rd, & 4th weeks on diets	85.00 ±4.77	80.72 ±2.09	86.00 ±2.35	96.00 ±4.20	90.00 ±5.56	37.86 ±5.25	56.11 ±2.62	61.36 ±2.09	71.88 ±2.43	83.39 ±1.07
Methionine rat series (choline-free diets)										
Methionine in diet (g./kg.)	1.6	3.0	8.0			1.6	3.0	8.0		
Choline equivalent for labile methyl	0.4	0.75	2.0			0.4	0.75	2.0		
Deaths on 8th day	1/12	1/12	0/12			2/12	2/12	0/12		
Weight gain (g.) during 2nd, 3rd, & 4th weeks on diets	81.36 ±3.05	82.00 ±3.77	85.83 ±0.77			46.00 ±1.57	66.11 ±2.62	75.83 ±2.27		
Caffeine rat series (choline-free diets)										
Citrated caffeine in diet (g./kg.)	0.8	1.5	4.0	8.0		0.8	1.5	4.0	8.0	
Choline equivalent for labile methyl	0.2	0.4	1.0	2.0		0.2	0.4	1.0	2.0	
Deaths on 8th day	11/12	6/12	0/12	0/4		6/12	2/12	0/12	0/4	
Weight (gain (g.) during 2nd, 3rd, & 4th weeks on diets		56.67 ±4.05	58.33 ±3.53	32.50 ±3.57		35.00 ±1.41	30.00 ±1.00	41.67 ±1.23	45.83 ±2.25	

In our experience, half to three quarters of all weanling rats placed on

* RH = relative humidity.

choline-free diets die from acute hemorrhagic nephritis on the eighth or ninth day. Methionine seemed slightly less effective than choline in preventing these deaths in both heat and cold, considering the two substances on the basis of their labile-methyl equivalents. Caffeine gave about the same degree of protection in the heat but somewhat less in the cold. No deaths from hemorrhagic nephritis occurred when the diet contained as much as 0.4% of citrated caffeine.

Methionine seemed well able to replace choline in supporting normal growth, either in the heat or in the cold. Optimal growth was achieved with it at approximately the same labile-methyl equivalent as with choline, and this was true with the high requirements of the hot room as well as in the cold. However, the story was quite different with caffeine. Rats on the higher amounts of caffeine were nervous, jumpy, and restless. Two of the four cold room rats on 8 g./kg. died late in the second week; the remaining two appeared scrawny and somewhat spastic in their movements about the cage. The hot room rats on the higher amounts of caffeine appeared normal in all respects except for their retarded growth.

It appears evident, therefore, that caffeine cannot be used as a choline substitute in meeting the high labile-methyl needs for growth in tropical heat. This may be because of its excessive stimulation of the central nervous system in the amounts required to supply the needed labile-methyl.

Conclusions

Methionine seems able to replace choline in preventing acute hemorrhagic nephritis in weanling rats and in supporting subsequent normal growth in either hot or cold environments.

Caffeine gives somewhat poorer protection against hemorrhagic nephritis and much poorer support to subsequent growth. It appears more toxic in the cold than in the heat, and this toxicity may be responsible for the poor growth at high intake levels.

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N.B. The B-vitamins used in this study were very kindly supplied by Merck & Co., Inc., the Haliver Oil by Abbot Laboratories, and the methionine by the Research Laboratories of S.M.A. Corporation.

Studies on the Source of Serum Phosphatase

The Nature of the Increased Serum Phosphatase in Rats after Fat Feeding

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Introduction

The source of serum 'alkaline' phosphatase has been the subject of numerous investigations. Armstrong and Banting (1) showed that extirpation of various organs of dogs does not lower the serum phosphatase level but may actually increase it and suggest that bone is the sole source of the enzyme. Kay (2) has observed that certain bone diseases in man result in highly elevated serum phosphatase levels and was led to conclude that the bone is a major source of the serum enzyme. Gould and Shwachman (3) have come to a similar conclusion from a study of the serum phosphatase in experimental scurvy where the decrease in activity parallels the cessation of osteoblastic activity of the guinea pig bone. A non-osseous source for serum phosphatase of dogs has been suggested by A. Bodansky (4) since he has observed elevated levels only after carbohydrate ingestion in young fasted animals. The influence of diet on serum phosphatase is also indicated by Weil and Russell (5), who find that in albino rats fasting lowers markedly the normally high phosphatase level and that the feeding of fat or certain fatty acids restores the phosphatase to its *normal* level. Bodansky and Jaffe (6) and Roberts (7) point to the liver as a source of serum phosphatase in view of the elevated levels observed in jaundice, and Bodansky (4) suggests that the normal serum phosphatase is probably of diverse origin and may be considered in bone disease to be of osseous origin, in liver disease of hepatogenous origin, and after carbohydrate ingestion to be of mixed origin. Kay (8) considers

the phosphatase of normal serum to be of diverse origin and due to "leakage" from tissues of high content (bone, kidney, and intestine).

In the course of investigations in this laboratory it was found that prolonged fat feeding to fasted rats resulted not merely in the restoration of normal levels, as reported by Weil and Russell (5), but rather in the production of highly elevated phosphatase levels. Experiments are described to indicate that the increase is actually the result of increased enzyme concentration and that the enzyme does not appear to be of bone or kidney origin but probably of intestinal origin.

EXPERIMENTAL

Animals. Albino rats weighing from 150–200 g. were used throughout in these experiments. The normal diet consisted of Purina checkers which were fed *ad libitum.* Water was available at all times. Other substances were fed by stomach tube and supplies were available in the food containers at all times.

Phosphatase Estimation. The serum phosphatase was estimated essentially by the micro-method of Shinowara, Jones, and Reinhart (9). Blood was drawn (about 0.2 ml.) directly from the heart under light ether anaesthesia and allowed to clot. The serum was collected and used for analysis. Tissues were excised, washed carefully, and then ground in water saturated with $CHCl_3$. The brei was allowed to autolyze at room temperature for 24–48 hours and estimations were carried out on the supernatant solution which was diluted to the proper range of activity. All results were calculated in terms of phosphatase units, each equivalent to 1 mg. of phosphorus, as phosphate, liberated by 100 cc. of serum (or 100 mg. of tissue, dry weight) in 1 hour at 37°C. under the conditions of the estimation.

The substrate consisted of 0.5% Na-β-glycerophosphate (E.K.) in 0.42% sodium diethyl barbiturate (veronal) (Merck) and 0.2% magnesium chloride. After the addition of serum the digest was at pH 8.6. The phosphorus was estimated colorimetrically in a Hilger photoelectric absorptiometer.

Influence of Fat Feeding. The serum phosphatase activity of a group of rats weighing about 150–200 g. each which had been on an exclusive diet of whole powdered milk was determined. It was found that the levels were extremely elevated as compared to those of animals fed a diet of animal feed* (Table I). Experiments were undertaken to determine the component of the milk diet which is responsible for the elevated phosphatase activity. Casein, skim milk, and butter fat were fed to animals and from the results (Table I) it is apparent that the fat is responsible for the increase in serum enzyme. Weil and Russell (5) have previously reported the influence of fat in restoring the lowered phosphatase level that results after fasting, but no observations of highly elevated levels were reported.

* Purina Chow.

This may be due to the fact that high fat feeding was not continued by them for sufficiently extended periods.

Experiments were then carried out in which animals previously fasted for 48 hours were fed various natural fats, or sodium oleate by stomach tube for 3 to 6 days (fats and oleate were also available in cages at all times) after which phosphatase estimations were carried out. The responses after shorter intervals are not reported except in special cases since our observations confirm those of Weil and Russell (5). The results indicate extremely elevated phosphatase levels after extensive fat feeding for all the fatty substances tested (Table I). Fasting of these animals

TABLE I

Influence of Fat Ingestion on Serum Phosphatase

Diet	Animals	Phosphatase Values	Phosphatase
	Number	*Range*	*Average*
Stock Diet	17	37.4–121	78
Milk powder	16	83 –261	151
Casein, 3 days	3	51 –109	79.5
Butter fat, 24 hrs.	3	71 –156	111
Butter fat, 3 days	6	116 –267	217
Sodium Oleate, 3 days	5	93 –164	140
Sodium Oleate, 6 "	5	153 –292	199
Olive oil, 3 days	3	284 –333	315
Cod liver oil, 3 days	6	150 –446	285
Corn oil, 3 days	5	184 –408	272

resulted in very low serum phosphatase levels only after several days. Normal animals show a sharp decline 12–24 hours after food is withdrawn.

Proof of Quantitative Alteration in Enzyme. In order to prove that there is an actual quantitative change in the phosphatase of the serum rather than a change due to the presence of some activator after fat feeding or the presence of an inhibitor after fasting, a series of experiments was undertaken in which mixtures of sera of contrasting activity, in varying proportions, were studied. Using sera with activities ranging from 9.6 units to 446.0 units it was found (Table II) that the observed phosphatase values compared favorably with the calculated values. This would strongly suggest that there is actually a quantitative change in the serum phosphatase both after fasting and after intensive fat feeding.

Characterization of Rat Serum Phosphatase by Bile Salt Inhibition.

In view of the difference in inhibition of bone and kidney phosphatase on the one hand, and intestinal phosphatase on the other by bile salts (10), a series of experiments was undertaken in which the degree of inhibition by sodium taurocholate (Merck) of the phosphatase activity of sera of animals fed a normal diet and sera from animals fed high fat diets was studied. The taurocholate was made up fresh and added to the substrate (5.15 mg. per ml.) immediately before incubation with the enzyme. A series of experiments was also carried out in which intestine, bone, and kidney autolyzates were studied in the same way. The results of the experiments are shown in Table III. In both the normal and fat-fed animals the inhibition does not resemble that for either bone or kidney

TABLE II

Addition Experiments: Mixtures of Active and Inactive Sera

Sera	Observed Phosphatase *units*	Calculated Phosphatase *units*
(1) A + B	79.0	87.0
(2) A + B	70.0	64.2
(3) A + B	114.0	109.0
(4) C + D	185.0	170.0
(5) E + F + G	143.0	138.0
(6) H + I	240.0	221.0
(7) K + L	28.2	23.0
(8) K + L	16.5	16.5

A, 9.6; B, 164; C, 31.5; D, 446; E, 333; F, 199; G, 31.7; H, 89; I, 179; K, 119; L, 47 phosphatase units.

phosphatase and indicates that the enzyme is not of such origin. The degree of inhibition is, however, strikingly similar to that observed with intestinal phosphatase. Inhibition studies cannot be interpreted with liver preparations in view of the high concentration of bile salt normally present.

Serum Phosphatase of Young Rats. Folley and Kay (11) have pointed out that increased phosphatase characterized growing bone in the young which is usually reflected in high serum enzyme levels. It might be expected that the young rat would contain phosphatase of osseous origin in the serum. A group of young rats was put on a normal diet, and serum phosphatase estimations were carried out periodically both before and after fasting. In the younger animals (Table IV) the residual phosphatase after fasting shows a higher inhibition with taurocholate, indicating

an osseous phosphatase component in the serum during early growth. Unlike other species studied, young rats have lower serum phosphatase levels than do full-grown animals, indicating a non-osseous source for the bulk of the serum enzyme in the adult. This view is further strengthened

TABLE III

Sodium Taurocholate Inhibition of Blood and Tissue Phosphatase

Diet	Sera or tissues *Number*	Inhibition *per cent*
Purina chow	12	26.0
Milk powder	11	22.7
Butter fat	6	18.0
Sodium oleate	6	27.8
Olive oil	6	32.9
Fasted 24 hours	11	25.4
" 48 hours	6	45.1
" 72 hours	5	40.9
Tissues		
Intestine (Duodenum)	9	21.1
" (Ileum)	6	38.1
Bone	8	69.9
Kidney	8	70.7

TABLE IV

Influence of Taurocholate on Serum Phosphatase of Young Animals

Animals	Age *days*	Number	Phosphatase *units*	Taurocholate Inhibition *per cent*
Normal	15	5	37.0	29.6
Fasted 48 hrs	17	5	13.4	66.1
Normal	30	3	81.0	22.3
Fasted 48 hrs	32	4	22.3	46.5
Normal	Adult	12	80.0	26.0
Fasted 24 hrs	Adult	11	27.9	25.4
" 48 "		6	19.9	45.1

by the normally high phosphatase level in the rat as compared to other animals and the difference in taurochloate inhibition (Table V).

Tissue Phosphatase of Normal, Fasted, and Fat-fed Animals. A series of estimations were carried out to determine whether there were marked alterations in the tissue phosphatase content of fasted or fat-fed animals from that of normal animals. It might be expected that the elevated level of the enzyme in the blood might be accompanied by an elevated level at

the center of production. The tissues of highest activity as well as the liver, which normally is of low activity, were selected for study. The kidney, intestine (at several levels), bone, and liver were excised and prepared as described. The results were calculated on the basis of the dry weight of the tissue. Under the conditions no significant variation from the normal enzyme content was observed.

The inability to demonstrate a marked change in the phosphatase activity of the tissues studied is not inconsistent with the hypothesis that the highly elevated serum phosphatase level after fat feeding is merely a matter of increased absorption of the enzyme into the blood from a tissue of normally high enzyme level, perhaps the intestine. The actual amount of enzyme required to elevate the serum level from 80 units to 300 units, where each unit is equivalent to mg. P liberated by

TABLE V

Sodium Taurocholate Inhibition of Serum Phosphatase

Animal	Number	Phosphatase *Average*	Inhibition *per cent*
Guinea pig (young)	(10)	12.0	54.2
Rabbit	(3)	5.0	50
Dog	(6)	4.0	64
Rat	(12)	80 0	26.0
Human (children)	(6)	8 7	45.3
Rooster	(1)	13 2	50 0
Pigeon*	(1)	8 7	0.0

* Blood serum highly jaundiced

100 cc. of serum, is relatively small in view of the small blood volume of the rats used (approx. 10.5 cc.) (12) and amounts to 23.5 units. From the tissue analyses of the intestinal tract of fasted, normal, and fat-fed animals 23 samples of duodenum had an average of 30 units per 100 mg. tissue (dry weight), and 39 samples of jejunum and ileum had an average of 10.4 units per 100 mg. tissue (dry weight). Assuming the total weight of the alimentary tract as 10 g. of which 25 per cent is dry weight (12) the total phosphatase of the rat intestine should contain approximately 275 units. It is obvious that a decrease of 23.5 units or less would fall within the variability of the estimations.

If a similar calculation is made for the liver whose average phosphatase level (22 samples) was found to be 0.41 mg./100 mg. tissue (dry weight) it is found that, assuming an average dry weight of 2.6 g. for the liver

(12), the *total* phosphatase activity is 10.7 units which is considerably less than would account for the increase in the blood. If an organ of low activity, such as the liver, were responsible for the increase in the blood enzyme level it might be expected that its normally low level would be elevated. However, there still remains the possibility that any overproduction is at once diverted to the blood.

Summary

Prolonged feeding of fat to rats results in an increase in the serum phosphatase to extremely high levels.

The increase in serum phosphatase after fat feeding and the decrease after fasting are due to a quantitative alteration in the enzyme rather than to the action of an inhibiting or activating agent.

The increased serum phosphatase does not appear to be of bone or kidney origin but may be of intestinal origin.

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Horse Brain Thromboplastin as a Reagent for the Quantitative Determination of Prothrombin*

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Introduction

Thromboplastin prepared from rabbit brain is used widely as a reagent for the quantitative determination of prothrombin by the method of Quick (16, 17). However, the preparation of large quantities of rabbit brain thromboplastin is difficult, since the amount of material obtained from one animal is small. For example, using Quick's method, one rabbit brain will yield approximately 16 cc. of thromboplastin suspension. It would be desirable to prepare thromboplastin from the brain of a larger animal, provided the activity of the preparation approximated that of rabbit brain. Most animal brains are not active enough to compete with rabbit brain. Some of the clotting times reported in the literature for normal human plasma are listed in Table I. These values were obtained by the method of Quick (or by minor modifications of it) with thromboplastins prepared from the brains of various animals. Included for comparison are some preparations made in this laboratory. Of the large brains listed, only human and horse brains appear to be sufficiently active to be used as reagents for prothrombin time determinations. Human brain has been employed for this purpose (5, 10, 20). Obviously it is not an easily available tissue. Horse brain thromboplastin appeared sufficiently promising to warrant investigation.

Apparently, no reports of the use of horse brain thromboplastin as a reagent for the quantitative determination of prothrombin have appeared in the literature. Quick (18) referred to the activation of the

* The data in this paper were presented at a meeting of The Physiological Society of Philadelphia on December 21, 1943.

prothrombin of horse plasma by the thromboplastin prepared from the same species. Actual clotting times were not reported. Aggeler and Lucia (1), who were interested in testing the potencies of a number of thromboplastins designed for local and hypodermic administration, found that horse brain thromboplastin clotted recalcified normal human plasma in 24 seconds by the Quick test when the thromboplastin was diluted to optimal activity. No other references to horse brain thromboplastin have been found.

Some of our preparations of horse brain thromboplastin are somewhat more active than those of Aggeler and Lucia. We have studied the suitability of these preparations as substitutes for rabbit brain thromboplastin in the Quick test. First, the optimal concentrations of reagents employed in the test were determined when rabbit brain thromboplastin was replaced by that of horse brain. With this information, normal prothrombin curves were prepared with both thromboplastins. Using these standard curves, the prothrombin concentrations of plasmas of known and unknown prothrombin contents were determined with each thromboplastin, and the results were compared.

Experimental Results

The Influence of the Concentrations of Calcium Chloride and of Thromboplastin on the Activity of Horse Brain Thromboplastin in the Quick Test. In the Quick test (17), 0.1 cc. of plasma is mixed with 0.1 cc. of thromboplastin suspension at 37°C., and the mixture is recalcified with 0.1 cc. of 0.277 per cent calcium chloride solution. When normal human plasma and horse brain thromboplastin were tested with this concentration of calcium chloride, clotting times of 23.3 to 27.3 seconds were obtained. However, if the concentration of calcium chloride was varied between 0.507 per cent and 0.092 per cent, the other reagents remaining constant, shorter clotting times were observed with lower concentrations of calcium chloride.

Fig. 1 shows the changes graphically for one preparation. A minimum in the clotting time appears in the neighborhood of 0.184 per cent calcium chloride. This was chosen as the optimal concentration for the remainder of the study. The concentration of calcium chloride suggested by Quick gave clotting times higher by 1.6 to 4.9 seconds.

When the concentration of calcium chloride was held constant at 0.184 per cent, and the concentration of horse brain thromboplastin was varied by dilution with distilled water or oxalated saline solution, the

clotting times of normal human plasma were shortened still further. Results were obtained for ten preparations, two of which were diluted with oxalated saline solution (2 per cent of 0.1 *M* sodium oxalate in 0.85 per cent sodium chloride) and the remainder with distilled water. Minimal clotting times were observed for eight of the ten thromboplastin preparations when they were employed in concentrations of 50 per cent of the original suspension as prepared by Quick's procedure. The minimal

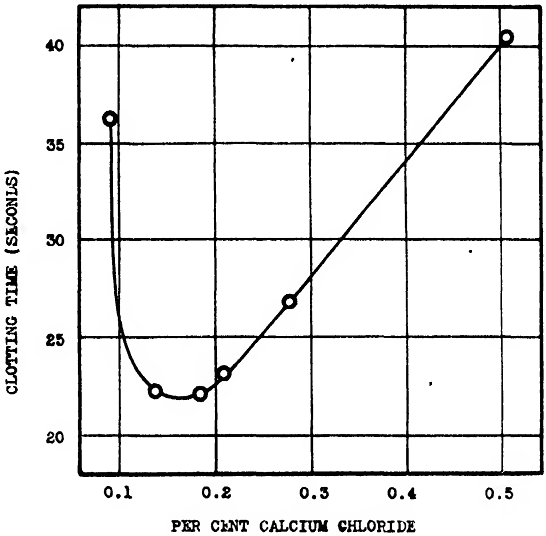

FIG. 1

The Effect of Calcium Chloride Concentration on the Clotting Time of Citrated Human Plasma as Determined by the Method of Quick Using Horse Brain Thromboplastin

zone was much broader than that produced by changing the concentration of calcium chloride. In order to insure the presence of sufficient thromboplastin, it was decided to use a concentration of 50 per cent for our tests, even though in some cases the material could be diluted further with little change in clotting time. In the case of two preparations, dilution of the thromboplastin did not shorten the clotting time significantly. The question of choice of diluent for the thromboplastin has not been studied extensively, but the data furnished by one sample appeared to indicate that distilled water is preferable to oxalated saline solution.

Comparison of Rabbit and Horse Brain Thromboplastins as Reagents for the Quick Test. Prothrombin curves were prepared for one sample of human plasma, using both horse and rabbit brain thromboplastins as reagents. The plasma was diluted with prothrombin-free plasma to 70, 50, 40, 30, 20, and 10 per cent of the original concentration. These standard plasma solutions were held in the frozen state at $-20°C.$ so that both thromboplastins could be checked periodically with the same plasma used to construct the original prothrombin curves. The prothrombin content of these solutions remained unchanged over a period of four months.

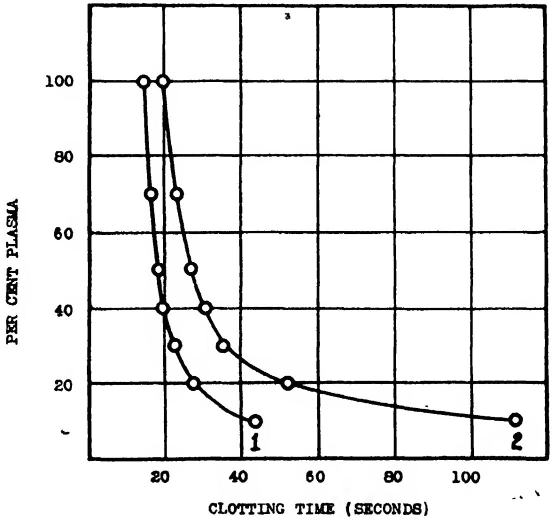

FIG. 2

Standard Prothrombin Curves
1. Rabbit brain thromboplastin.
2. Horse brain thromboplastin.

The standard prothrombin curves are presented in Fig. 2. Curve 1 was obtained with undiluted rabbit brain thromboplastin and 0.184 per cent calcium chloride, and Curve 2, with 50 per cent horse brain thromboplastin (diluted with distilled water) and 0.184 per cent calcium chloride solution. Both samples of thromboplastin had been lyophilized. A concentration of 0.184 per cent calcium chloride was used with the rabbit brain thromboplastin because it gave faster clotting times than did the

concentration of 0.277 per cent originally suggested by Quick. The former concentration appears to be very nearly optimal and gives about the

TABLE I

Clotting Times of Normal Human Plasma Obtained with Various Thromboplastin Preparations by the Method of Quick

Source of Thromboplastin	Clotting times *seconds*	Investigators
Rabbit brain	10–11.5	Quick (19)
	10	Pohle and Stewart (14)
	12–14	Norris and Bennett (11)
	13.9[a]	Owen and Toohey (13)
	12–18	This laboratory
	14–20	Copley (3)
	18	Poncher, Ricewasser, and Kato (15)
	19.7[a]	Hause and Tocantins (4)
	19–25[a]	Kark and Souter (6)
	19–24	Kaump and Greenwood (7)
	23.4[a]	Kelley and Bray (9)
Human brain	12	Tocantins (20)
	20–40[a]	Illingsworth (5)
	25–30[a]	Larsen and Plum (10)
Horse brain	16.8–25.8	This laboratory
	24[a]	Aggeler and Lucia (1)
Dog brain	25[a]	Aggeler and Lucia (1)
Calf brain	32[b]	This laboratory
	34[a]	Aggeler and Lucia (1)
Guinea pig brain	35	Quick (19)
	113[a]	Aggeler and Lucia (1)
Hog brain	36[b]	This laboratory
Chicken brain	45	Quick (19)
Sheep brain	90[a]	Aggeler and Lucia (1)

[a] Not dehydrated with acetone before extraction with saline solution.
[b] Prepared from tissue several hours old.

same clotting times as the 0.22 per cent (0.02 M) concentration later recommended by Quick (19).

TABLE II

A Comparison of Horse and Rabbit Brain Thromboplastins by a Quantitative Determination of Prothrombin

Thromboplastin	Observed clotting times	Theoretical prothrombin content	Observed prothrombin content	Deviation from theoretical	Difference between thrombo-plastins
1. With dilutions of human plasma of known prothrombin concentration					
	seconds	*per cent*	*per cent*	*per cent*	*per cent*
Rabbit	14.5	100	99.0	−1.0	
Horse	19.5	100	99.0	−1.0	0.0
Rabbit	15.0	85	90.0	+5.9	
Horse	21.1	85	85.5	+0.6	5.5
Rabbit	17.1	60	63.5	+5.8	
Horse	23.7	60	65.0	+8.3	2.4
Rabbit	20.5	35	36.5	+4.3	
Horse	32.2	35	35.5	+1.4	2.8
Rabbit	36.2	15	12.3	−18.0	
Horse	70.2	15	12.0	−20.0	2.4
2. With human plasmas of unknown prothrombin concentration					
	seconds	*per cent*	*per cent*	*per cent*	*per cent*
Rabbit	14.6	100	97.0	−3.0	
Horse	19.5	100	99.0	−1.0	2.1
Rabbit	15.7	Sample 1	81.0		
Horse	19.5		86.0		6.2
Rabbit	17.3	Sample 2	61.5		
Horse	23.4		67.5		9.7
Rabbit	23.2	Sample 2	29.0		
Horse	37.0	(50 per cent)[a]	28.5		1.7
Rabbit	16.4	Sample 3	72.0		
Horse	21.5		81.0		12.5
Rabbit	16.7	Sample 3	69.0		
Horse	21.9		78.0		13.0
Rabbit	22.7	Sample 3	29.5		
Horse	30.8	(50 per cent)[a]	38.5		30.5

[a] Diluted with prothrombin-free plasma.

The reproducibility of these curves was checked by preparing dilutions of the same plasma used to construct the curve, and determining the clotting times of these dilutions with the original reagents. The clotting times obtained were converted to prothrombin concentrations with the aid of the standard curves for the respective thromboplastins (Fig. 2). A comparison then was made of the observed and theoretical prothrombin concentrations. The results are given in Table II, Part 1. The differences between the observed prothrombin values obtained with each thromboplastin for any one dilution were less than 6 per cent. Except at very low levels of prothrombin, the greatest difference between the observed and theoretical prothrombin concentrations was less than 9 per cent for either thromboplastin.

The prothrombin contents of various lots of presumably normal plasma, whose prothrombin contents were not actually known, also were determined. Clotting times were obtained with each thromboplastin as already described, and were translated to prothrombin concentration from the standard curves (Fig. 2). These results are presented in Table II, Part 2. With a single exception, the observed prothrombin concentrations obtained with each thromboplastin agreed within 13 per cent. Controls made with the plasma employed in obtaining the data recorded in Part 1 of Table II showed that the thromboplastic activity was unchanged. The prothrombin levels of these samples were below that of the control, even though they had been obtained from pooled lots of apparently normal plasma. The results appear to indicate that the variations of the determination are just as great for rabbit brain as for horse brain thromboplastin.

Discussion

The thromboplastin preparations made from horse brain clotted recalcified human plasma in 16.8 to 25.8 seconds when the optimal concentrations of calcium chloride and thromboplastin were used in the Quick test. The optimal calcium chloride concentration (*i.e.*, the concentration producing the fastest clotting time) for the plasma used was found to be 0.184 per cent. It is not known whether the calcium chloride influences the thromboplastin or other factors. Probably the optimal calcium chloride concentration is a function of the anticoagulant concentration present in the plasma, and is not influenced by the thromboplastin. This concept is supported by the finding that entirely unrelated thromboplastin preparations, such as those prepared from hog and calf

brains, also have a minimal clotting time in the neighborhood of 0.184 per cent calcium chloride when tested with our plasma.

Still shorter clotting times for optimally recalcified plasma could be obtained by using horse brain thromboplastin suspensions that had been diluted to 50 per cent and 25 per cent of the original concentration as prepared according to Quick's directions. Since dilution with water to concentrations less than 50 per cent did not improve the clotting times, this concentration was chosen as desirable. Further dilution reduced the solid content to a point where it became difficult to determine the exact time of clotting. This peculiar effect of increased activity with dilution of horse brain thromboplastin has been demonstrated also by Aggeler and Lucia (1). According to Nygaard (12), however, rabbit brain thromboplastin does not act in this manner, dilution actually causing a slight prolongation of the prothrombin time in the region of 25 per cent thromboplastin. On the other hand, we have observed no such dilution effect for two of the ten extracts, both of which were prepared from the acetone-dried powder of the same brain. Perhaps in these cases inhibitors of the clotting mechanism were not extracted from the acetone-dried brain powder in sufficient concentration to exert an anticoagulant action. Apparently the remaining preparations contained inhibitors whose influence on the rate of the clotting reaction could be nullified in part by dilution. That such inhibitors exist in brain tissue has been demonstrated by Chargaff (2), who showed that they may be found in the cerebroside fraction of sheep and pig brains. Incidentally, these inhibitors were insoluble in acetone and hence might be present in the acetone-dried powders prepared by Quick's procedure.

The presence of acetone-soluble inhibitors in fresh brain has been demonstrated by Quick (19, page 65), who showed that the activity of most thromboplastin preparations is increased by a preliminary treatment of the fresh brain with acetone. Quick (16) recommends an acetone dehydration period of ten minutes. In one of our experiments it was demonstrated that a longer period of treatment produced a more-active horse brain thromboplastin. Two acetone-dried powders were prepared from the same brain, with the same technic, except that one (6A-2) was subjected to a total acetone extraction period of 25 minutes, while the other (6B-2) was subjected to the usual overnight extraction employed in this study. The 6A-2 preparation clotted human plasma in 21.4 seconds, while the 6B-2 clotted the same plasma in 18.8 seconds, at optimal

concentrations of calcium chloride and thromboplastin. Apparently acetone-soluble inhibitors were removed by the longer treatment with acetone.

A comparison of the normal prothrombin curves makes it evident that rabbit brain thromboplastin is more active than is horse brain thromboplastin. This is evident especially in the region of low prothrombin concentrations, where the two curves become widely divergent. Both of these curves, however, can be reproduced with the same degree of accuracy. When various known dilutions of the same plasma used to construct the curves were assayed from time to time, the observed prothrombin values differed from the theoretical values for each thromboplastin by not more than 9 per cent. Furthermore, results obtained with both thromboplastins agreed within 6 per cent. Admittedly this was an "ideal" test of the method, since the plasma tested was the same as that used to construct the standard curves. However, it did indicate that the standard curves prepared with each thromboplastin were reproducible with the technic employed.

A test designed to indicate whether horse brain thromboplastin can replace the usual rabbit brain material in the Quick procedure was made with plasmas of unknown prothrombin contents. In six of seven assays, rabbit and horse brain thromboplastins gave results that agreed within 13 per cent. In this series horse brain thromboplastin usually gave slightly higher results than did rabbit brain thromboplastin, whereas in the series mentioned above the reverse was true. Unfortunately, a comparison on the basis of the theoretical amount of prothrombin present in these samples could not be made; hence it could not be decided which thromboplastin gave results most closely approximating the true values. In general, however, the data indicate that horse brain thromboplastin can be used instead of rabbit brain thromboplastin as a reagent for the Quick test under the conditions employed in these experiments. A comparison of both thromboplastins as reagents for the analysis of plasmas from hospital patients would be desirable. Such a study might indicate more specifically whether or not horse brain thromboplastin will be useful clinically.

Some of the thromboplastin preparations used in these experiments were lyophilized. Most of these showed no change in potency before and after drying by this technic; some showed a slight loss of potency, usually of the order of one second. Our oldest lyophilized preparation (under vacuum at 5°C.) has shown no loss of potency over a period of 14 months.

Experimental Details

Preparation of Acetone-Dried Brain Powders. Rabbit brain thromboplastin was prepared by the method of Quick (16). For the preparation of horse brain thromboplastin, Quick's technic was modified slightly in order to simplify the handling of larger amounts of material. The warm brain of the horse was washed with running tap water and was stripped as completely as possible of its pia and blood vessels. Excess water was removed with blotting paper. The washed tissue then was macerated in acetone (U. S. P. grade) with the aid of a Waring blendor; small portions of tissue were treated with an excess of acetone until all of the brain was finely minced. Sufficient acetone was then added to the suspension to make a total volume of 8 to 12 liters. The mixture was stirred and allowed to settle out during a period of one hour. The supernatant liquid was siphoned off and discarded. A second extraction was made with 8 to 12 liters of acetone overnight at 5°C. On the following morning, the second extract was discarded and the residue was washed twice with 4 to 6 liters of acetone, filtering quickly after each washing through Whatman No. 5 paper on Buchner funnels (230 mm. diameter) with suction. After the second washing, the material was left on the filters and most of the acetone was "squeezed out" with the aid of suction and a rubber dam. At intervals the residue on the filters was mixed with a spatula and "squeezed out" each time during a period of thirty minutes. This procedure usually dried the residue completely; occasionally, more than thirty minutes were necessary. As an extra precaution, the light, fluffy crude acetone-dried brain powder was dried in a vacuum oven at room temperature overnight. It was then passed through a 20-mesh sieve (on a shaking machine) in order to remove strands of fibrous material, which comprised 11 to 12 per cent of the crude powder. The resulting powder was stored at $-20°$ C. in tightly stoppered jars. The yields of the final product were 10 to 16 per cent of the weight of fresh tissue. The fresh horse brains weighed from 570 to 740 g.

Preparation of Saline Extracts. Saline extracts of both rabbit and horse brain powders were prepared by a method differing only in minor details from that described by Quick (16). For example, 15 g. of the acetone-dried horse brain powder were suspended in 250 cc. of oxalated saline solution (2 per cent by volume of 0.1 *M* sodium oxalate in 0.85 per cent sodium chloride solution) with the aid of a Waring blendor* (less than one minute), and was heated for fifteen minutes at 50° C. in a water bath. The suspension was cooled to room temperature under tap water, and was centrifuged at 1700 r. p. m. for ten minutes. The residue was then washed with a volume of oxalated saline solution equivalent to the difference between the original volume of the saline solution used and the volume of extract obtained after centrifugation. The two extracts were combined, a portion was withheld for assay, and the remainder was lyophilized in small vials containing 1 to 5 cc. of suspension.

Prothrombin Determinations. Clotting times were determined by the method of Quick (17), except for the indicated changes in the concentrations of calcium chloride and thromboplastin. All samples of plasma were obtained from pooled

* The Waring blendor was not employed for the preparation of the saline extracts of acetone-dried rabbit brain powder.

lots of human bleedings used for the commerical production of plasma. The anticoagulant used was sodium citrate (50 cc. of 4 per cent sodium citrate solution for each 500 cc. of blood). These samples were approximately three days old when received, and were used immediately, or frozen rapidly and stored at −20° C., or preserved by the lyophile technic (8). For the construction of normal prothrombin curves, dilutions of plasma were made with prothrombin-free plasma prepared by filtration of normal plasma through a Seitz K-3 pad. Such filtered plasma did not clot in the presence of thromboplastin and calcium chloride (Quick test) over a period of 24 hours. It contained sufficient fibrinogen, as indicated by the abundant precipitation induced by one-fourth saturation with ammonium sulfate, and by the firm clot formation caused by the addition of thrombin.

Summary

1. Preparations of horse brain thromboplastin clotted human plasma in 16.8 to 25.8 seconds when optimal concentrations of calcium chloride and thromboplastin were used in the Quick test. The optimal concentration of calcium chloride was 0.184 per cent for the plasma used by us. Dilution of the brain suspensions prepared according to Quick's directions with an equal volume of water yielded preparations that gave minimal clotting times.

2. Although rabbit brain thromboplastin is more active than is horse brain thromboplastin, it appears that the horse brain preparation is suitable for the quantitative determination of prothrombin. With plasmas of known prothrombin contents, determinations usually could be made to within 9 per cent of the theoretical concentrations of prothrombin. The differences in prothrombin concentration found with the two thromboplastins was not greater than 6 per cent. Except in one case, with plasmas of unknown prothrombin concentration the difference in concentrations determined with horse and rabbit thromboplastins was not greater than 13 per cent.

3. Horse brain thromboplastin has been lyophilized and then stored for 14 months with little or no loss of potency.

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The Study of Typhoid Antigens by Electrophoresis

I. Immunological Reactions

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Introduction

Recent advances in the study of antigens of the Gram-negative pathogens have been summarized by Morgan and Partridge (1) and Freeman (2) for typhoid bacilli, by Bornstein (3) for the Salmonella group, by Weil (4) for the dysentery organisms and by Linton (5) for the vibrios. While the antigens referred to in these papers were isolated by various methods from the organisms themselves, the typhoid antigens described in the present paper appeared in solution during growth of the organisms, and owing to the choice of a liquid medium containing only low molecular nutrient materials, they could be purified and concentrated without chemical treatment.

Materials and Methods

Three well-known strains of typhoid organisms were made available through the kindness of Dr. W. D. Stovall, Director of the State Laboratory of Hygiene, Madison, Wisconsin: H 901 (motile), O 901 (non-motile), and Ty 2, a non-motile strain containing the Vi antigen. A fourth, Ty 58, which also contains the Vi antigen and is widely used for vaccine, was obtained from the Army Medical School, Washington, D. C., through the courtesy of Major G. F. Luippold. These strains retained their serological character throughout the course of the work.

The simple medium designated 6a in earlier work (6) was aerated first with CO_2 for a few minutes and then with air. The heavy culture obtained after incubation for 24 hours was killed by the addition of 0.5 g. of phenylmercuric acetate to the 13 liters of culture. The sterile culture, neutralized if necessary, was concentrated *in vacuo* at about 40° to approximately one liter in twelve hours. The bacteria were removed in a Sharples centrifuge, and the remaining nutrient elements of the medium were eliminated by dialysis in Visking tubing for two days,

* Now in the Armed Forces.

first against tap water, then against distilled water. The solution was then further concentrated to about 100 ml. in the tubing by means of a current of warm air; the temperature of the solution itself remained below 24°. The resulting concentrate was finally cleared from any remaining bacteria and from most of the opacity by centrifuging in a multispeed attachment, with dry ice in the centrifuge casing to dissipate the heat generated.

Analysis of the Typhoid Concentrates by Electrophoresis

Each of the concentrated bacterial filtrates (hereafter referred to as concentrates) was centrifuged at high speed at low temperature in a Beams quantity air-driven centrifuge, and arbitrarily separated into an upper supernatant fraction, a lower supernatant fraction, and a sediment fraction (the gelatinous button deposited at the bottom of the tube). Both the unfractionated concentrates and the fractions thus prepared were examined by electrophoresis in the Tiselius apparatus (7) in a buffer solution of pH 6.9 containing 0.15 M NaCl and 0.02 M phosphate; a potential gradient of about four volts per centimeter was used throughout, and the boundaries were photographed by the Longsworth method (8). When maximum separation of the extreme boundaries had been effected, the contents of the various cell compartments were removed for the determination of protective and serological activity. All solutions were stored in the cold.

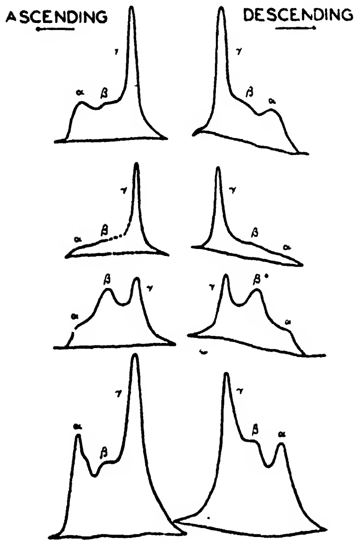

FIG. 1

Electrophoretic Patterns (Tracings of Longsworth Diagrams) for Typhoid Concentrates

After migration for one-half hour at a potential gradient of four volts per centimeter in a buffer solution of pH 6.9 containing 0.15 M NaCl and 0.02 M phosphate. From top to bottom: concentrate H, concentrate O, concentrate Ty 2, and concentrate Ty 58.

The high speed centrifuging was found to cause aggregation and interaction of the various constituents in the lower supernatant and sediment fractions. The tests of protective and serological activity were for this reason restricted largely to the fractions prepared by electrophoresis of the uncentrifuged concentrates. Aside from the vaccination tests, which are given in Table III, all the experiments with the fractions prepared by centrifugation will be reported in a subsequent paper (9).

Each concentrate contained three electrophoretic constituents, which

were designated alpha, beta and gamma in order of decreasing mobility (Fig. 1). The mobilities were similar but not identical from strain to strain, and the constituents were present in varying concentrations and proportions (Table I). None of the constituents appeared to be a fully

TABLE I

Analysis of Typhoid Concentrates by Electrophoresis

Buffer: pH 6.9, 0.15 *M* NaCl, 0.02 *M* phosphate. T: 0°C. Migration anodic

	Mobility × 10^5			Concentration,* mg./ml.			
Strain	*Alpha*	*Beta*	*Gamma*	*Alpha*	*Beta*	*Gamma*	*Total*
H	9.4	5.0	0.4	0.87	0.62	1.76	3.25
O	10.2	5.0	0.6	0.21	0.54	0.86	1.61
Ty 2	10.4	7.0	1.4	0.42	1.29	1.07	2.78
Ty 58	10.1	5.1	1.1	1.93	1.47	3.52	6.92

* Calculated from the areas of the electrophoretic diagrams on the assumption that the specific refractive increment of each constituent was 0.0018.

homogeneous entity, and in some instances the diagrams for longer periods of migration (Fig. 2) gave evidence for definite subconstituents (see also (9) and (10)). These subconstituents were not considered in the present treatment of the results because on the one hand the resolution of the boundaries was too poor to permit accurate estimates of the concentrations, and on the other hand the mouse protection experiments and the serological tests were not sufficiently precise to warrant a finer analysis.

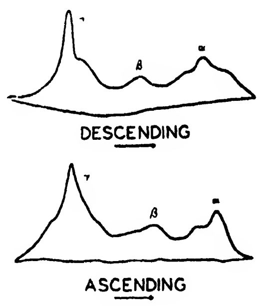

Fig. 2

Electrophoretic Pattern (Tracing of Longsworth Diagram) for Typhoid Concencentrate Ty 58

After migration for one and one-half hours at a potential gradient of four volts per centimeter in a buffer solution of pH 6.9 containing 0.15 *M* NaCl and 0.02 *M* **phosphate.**

The three main constituents appeared to migrate independently of one another in concentrates H, O, and Ty 2, while in concentrate Ty 58 there was evidence for the presence of reversibly dissociable complexes (11) of the beta constituent with the gamma and alpha constituents (Fig. 2). Later experiments with concentrate Ty 58, in which the concentration was varied by dilution or pressure dialysis, verified the formation of complexes and showed that as the volume was reduced, certain constituents were concentrated to the

point of saturation and precipitated. These findings, which are presented in more detail in the following papers (9, 10), are mentioned here because they appear to explain the otherwise anomalous results of some of the immunization experiments.

Immunization of Mice with Typhoid Concentrates and Fractions

A. *Concentrates.* The unfractionated concentrates of strains H, O and Ty 2 were arbitrarily diluted 1:10, and 0.1 ml. was injected intraperitoneally into Swiss mice weighing from 9 to 12 grams. Two weeks later the animals, which now weighed 15 to 18 grams, were injected by the same route with 0.5 ml. of a 5% mucin suspension containing approximately 100,000 typhoid organisms of the virulent strain 63. At the same time, three control groups consisting of mice which had been put aside for this purpose at the beginning of the experiment were injected intraperitoneally with approximately 1000, 100, and 10 virulent organisms in 0.5 ml. of 5% mucin. All experiments were terminated 72 hours after infection.

The H and O concentrates were less protective than the Ty 2 concentrate (Table II, Group A). The experiment was repeated (Table II, Group B), using equal amounts of material from each concentrate and the same test dose. While the death rate was lower than in the preceding experiment, Ty 2 concentrate again gave better protection than the H and O concentrates.

A more extensive experiment was carried out with a concentrate of Ty 58 which contained 6.9 mg. of dissolved material per ml. (Table II, Group C). It proved to be a highly effective agent, as little as 6.9×10^{-7} milligrams protecting some mice against 250,000 virulent organisms; it was thus capable of great dilution before its protective power was much diminished.

B. *Electrophoretic Fractions of Typhoid Concentrates.* The electrophoretic fractions from the typhoid concentrates were tested for immunizing potency under the same experimental conditions used for the unfractionated concentrates (Table III). The alpha and beta constituents of strain H were definitely not protective at the dilutions used. The corresponding constituents of the O strain protected mice at the 1:100 dilution but only partially at the 1:1000 dilution. The alpha and beta constituents of Ty 2 and Ty 58, either separate or combined, gave good protection at both of the dilutions used. The gamma constituents from the H and O strains were about as protective as their alpha and beta constituents. Both were inferior to the gamma constituent of the two virulent strains, Ty 2 and Ty 58. The electrophoretic fractions

showed the same variation from strain to strain as the unfractionated concentrates.

TABLE II

Immunization of Mice with Typhoid Concentrates

	Strain	Immunizing dose* in mg. Total	Alpha	Beta	Gamma	Infecting dose, Ty 63 organisms	Deaths 24 hrs.	48 hrs.	72 hrs.	Total
Group A	H	0.0325	0.0087	0.0062	0.0176	100,000	4	1	0	5/10
	O	0.0161	0.0021	0.0054	0.0086	100,000	3	1	0	4/10
	Ty 2	0.0278	0.0042	0.0129	0.0107	100,000	0	0	0	0/9
	Controls					1000	3	0	0	3/6
						100	5	0	0	5/8
						10	2	1	0	3/6
Group B	H	0.01	0.0027	0.0019	0.0054	100,000	1	0	0	1/6
		0.001	0.00027	0.00019	0.00054	100,000	5	1	0	6/6
	O	0.01	0.0013	0.0033	0.0054	100,000	1	0	0	1/6
		0.001	0.00013	0.00033	0.00054	100,000	2	0	0	2/6
	Ty 2	0.01	0.0015	0.0047	0.0038	100,000	0	0	0	0/6
		0.001	0.00015	0.00047	0.00038	100,000	0	0	0	0/5
	Controls					1000	2	0	0	2/3
						100	1	0	0	1/3
						10	1	0	0	1/3
Group C	Ty 58†	0.0692	0.0193	0.0147	0.0352	100,000	0	0	0	0/9
		0.00692	0.00193	0.00147	0.00352	100,000	0	0	0	0/14
		6.92×10^{-4}	1.93×10^{-4}	1.47×10^{-4}	3.52×10^{-4}	100,000	0	0	0	0/10
						250,000	0	0	0	0/6
		6.92×10^{-5}	1.93×10^{-5}	1.47×10^{-5}	3.52×10^{-5}	100,000	1	0	0	1/11
						250,000	1	0	0	1/6
		6.92×10^{-7}	1.93×10^{-7}	1.47×10^{-7}	3.52×10^{-7}	100,000	0	0	1	1/6
						250,000	3	0	0	3/6
	Controls					1000	8	1	0	9/16
						100	3	3	0	6/15
						10	1	1	0	2/15

* Calculated from the areas of the electrophoretic diagrams on the assumption that the specific refractive increment of each constituent was 0.0018.

† The resolution of the boundaries in concentrate Ty 58 was insufficient to permit the measured concentrations to be corrected for the effects of complex formation. As an approximation, the true concentrations were assumed to be given by the averages of the concentrations in the two arms of the U-Tube, since the differences were small. Because of the delta effect, the concentrations of the alpha and beta constituents were thereby somewhat underestimated, and that of the gamma constituent was somewhat overestimated.

Four immunization tests with constituents isolated from the sediment deposited by high speed centrifugation of the typhoid concentrates are

TABLE III

Immunization of Mice with Electrophoretic Constituents of Typhoid Strains

Infecting dose: 100,000 Ty 63 bacilli in 5% mucin

Strain	Immunizing dose*· † in mg.				Deaths			
	Total	*Alpha*	*Beta*	*Gamma*	*24 hrs.*	*48 hrs.*	*72 hrs.*	*Total*
H	0.01	0.0085	0.0015		3	1	0	4/5
	0.001	0.00085	0.00015		5	0	0	5/5
O	0.01	0.0091	0.0009		0	0	0	0/5
	0.001	0.00091	0.00009		2	0	1	3/5
Ty 2		0.01			0	1	1	2/7
			0.01‡		0	0	0	0/7
			0.001‡		0	0	0	0/7
	0.01	0.0072	0.0028		1	0	0	1/5
	0.001	0.00072	0.00028		0	0	0	0/5
Ty 58	0.01	0.0096	0.0004		0	0	0	0/6
	0.01	0.0053	0.0045	0.0002	2	0	0	2/7
	0.001	0.00053	0.00045	0.00002	2	0	0	2/7
H				0.01	2	0	0	2/4
				0.001	2	1	0	3/3
				0.01‡	8	0	0	8/13
				0.001‡	9	1	0	10/13
O				0.01	3	1	0	4/13
				0.001	4	2	0	6/10
Ty 2				0.01	0	1	0	1/12
				0.001	4	0	0	4/13
Ty 58	0.01		0.0002	0.0098	0	0	0	0/7
	0.001		0.00002	0.00098	1	2	0	3/8
	Infecting dose							
Controls	1000				6	1	0	7/12
	100				8	1	0	9/13
	10				1	3	0	4/12

* Calculated from the areas of the electrophoretic diagrams and the final positions of the electrophoretic boundaries on the assumption that the specific refractive increment of each constituent was 0.0018.

† The resolution of the boundaries in concentrate Ty 58 was insufficient to permit the measured concentrations to be corrected for the effects of complex formation. As an approximation, the true concentrations were assumed to be given by the averages of the concentrations in the two arms of the U-tube, since the differences were small. Because of the delta effect, the concentrations of the alpha and beta constituents were thereby somewhat underestimated, and that of the gamma constituent was somewhat overestimated.

‡ From sediment fraction.

reported in Table III. The changes produced by centrifuging (aggregation and interaction of the constituents) appeared to have little effect on the protective power.

None of the separate constituents of Ty 58 was quite as effective as the whole concentrate of Ty 58 from which it was derived. The evidence from electrophoresis that in concentrate Ty 58 reversibly dissociable complexes were formed by the beta constituent with both the alpha and the gamma constituents (10) suggests the existence of a complex of all three components, which may have been a more effective antigen than any one of the components alone. There is also a possibility that part of the difference in protective power may be accounted for by the following observations. Concentration of the medium after growth was found ultimately to produce saturation and precipitation of the bacterial products (9, 10). The resulting opacity was difficult to remove, and it was not taken into account in the concentrations calculated from the areas of the electrophoretic diagrams. The experiments referred to in Table II, Group C, were performed with a sample of the concentrate which had been centrifuged in a laboratory centrifuge only. The electrophoretic fractionation, on the other hand, was carried out with a sample which had been further centrifuged in a multispeed-head. The material thrown down by the multispeed attachment undoubtedly possessed antigenic activity, and its removal was at least partially responsible for the lower activity of the electrophoretic fractions.

Serological Examination of Fractions Obtained by Electrophoresis

The various fractions prepared by electrophoresis were tested serologically for their ability to precipitate with antisera to H, O, and Vi-containing strains of typhoid (Table IV). Precipitin tests were carried out by layering the diluted antigen solutions over equal quantities of antiserum, incubating for one hour at 37°, and examining the tubes for the presence of rings. The contents were then mixed, incubation was continued at 37° for another hour, and the tubes were placed in the refrigerator overnight. The amount of precipitate in each was noted the next morning.

Inhibition of agglutination was determined by mixing 0.1 ml. of antigen with decreasing amounts of immune serum. After the mixture had stood for fifteen minutes at room temperature, 0.5 ml. of suspension of O or Vi organisms were added to each tube. Agglutination was read after incubation for one hour at 37°.

The electrophoretic fractions of Typhoid H were tested for precipitin activity against H antiserum. The gamma constituent alone possessed

serological activity, since the lower positive fraction, which contained the alpha and beta constituents in full concentration and the gamma constituent in very low concentration, was inactive, while the upper

TABLE IV

Precipitin Activity of Fractions Prepared by Electrophoresis

Concentration	Fraction	Concentration* in mg./ml. *Alpha*	*Beta*	*Gamma*	Serological Activity			
					H Antiserum Ring	H Antiserum Precipitin		
H	Upper Positive	0.85	0.15	0	−	−		
	Lower Positive	0.85	0.61	0.29	−	−		
	Lower Negative	0.02	0.47	1.76	+++	+++		
	Upper Negative	0	0	1.46	+++	+++		
					O Antiserum Ring	O Antiserum Precipitin	Ty 2 Antiserum Ring	Ty 2 Antiserum Precipitin
O	Upper Positive	0.20	0.02	0	−	−	−	−
	Lower Positive	0.20	0.53	0.25	+	+	+	−
	Bottom Section	0.21	0.54	0.86	++	+	++	+
	Lower Negative	trace	0.48	0.86	++	+	++	+
	Upper Negative	0	0	0.58	++	+	++	+
					O Antiserum Ring	O Antiserum Precipitin	Ty 2 Antiserum Ring	Ty 2 Antiserum Precipitin
Ty 2	Upper Positive	0.37	0.14	0	+	−	+	−
	Lower Positive	0.41	1.26	0.30	++	+	++	++
	Bottom Section	0.42	1.29	1.07	++	+	++	+++
	Lower Negative	0.06	1.09	1.07	++	+	++	++
	Upper Negative	0	0	0.75	+	−	+	−
					H Antiserum Ring	H Antiserum Precipitin	Ty 2 Antiserum Ring	Ty 2 Antiserum Precipitin
Ty 58†	Upper Positive	1.52	0.06	0	+	−	+	−
	Lower Positive	1.75	1.40	0.05	+	++	+	+++
	Upper Negative	0	0.06	3.46	+	++	+	+++

* Calculated from the areas of the electrophoretic diagrams and the final positions of the electrophoretic boundaries on the assumption that the specific refractive increment of each constituent was 0.0018.

† The resolution of the boundaries in concentrate Ty 58 was insufficient to permit the measured concentrations to be corrected for the effects of complex formation. As an approximation, the true concentrations were assumed to be given by the averages of the concentrations in the two arms of the U-tube, since the differences were small. Because of the delta effect, the concentrations of the alpha and beta constituents were thereby somewhat underestimated, and that of the gamma constituent was somewhat overestimated.

negative fraction, which contained only the gamma constituent, was strongly active.

Similar tests were made with fractions obtained from an O strain, and the Vi-containing strain Ty 2. The inactivity of the alpha component of Typhoid O was shown by the failure of the upper positive fraction to cause precipitation. The inactivity of the beta component was shown by the slight response of the lower positive fraction with O serum and the negative precipitin reaction with Ty 2 serum; the precipitation of the O serum by this fraction must be ascribed to the presence of one-fourth the full concentration of the gamma component rather than to the full concentration of the beta component. It appears that in the fractions from this strain, as in those from the preceding strain, serological activity was associated entirely with the gamma component.

The tests with the fractions of Ty 2 showed that for this strain the serological activity was not restricted to the gamma constituent. The activity possessed by the upper positive fraction was due either to a very active beta component or to a less active alpha component. In an effort to determine the precipitinogen content of these fractions in a more nearly quantitative manner, dilutions were set up with Ty 2 antiserum. After one hour at 37° the precipitin tests were read, 1.6 ml. of normal saline was added to each tube, and the tubes were placed in the refrigerator. The next morning the turbidity of the contents of the tubes was determined in a photoelectric colorimeter, and expressed as the percentage absorption of light (Table V).

If we consider a turbidity value of about 17 as an end-point, then the bottom and lower positive fractions were about eight times as active as the upper positive. The lower positive fraction was much more active than its content of gamma component (0.30 mg./ml.) could account for, since the upper negative fraction, which contained more gamma component (0.75 mg./ml.), was only about one-fourth as active. The major portion of the activity must be ascribed, then, to the beta component. The alpha component can be ignored, for the upper positive fraction, which contained alpha component in almost full concentration, was no more active than could be accounted for by the small amount of the beta component.

Evidently in the Vi-containing Ty 2 strain, both the beta and the gamma components were active, in contradistinction to the H and O strains previously tested. The lower positive, bottom, and lower negative fractions were found to inhibit the agglutination of Ty 2

organisms by Ty 2 antiserum. Since this agglutination is governed by the presence of Vi antigen in the organisms being agglutinated, Vi antigen was apparently present in these electrophoretic fractions.

The results of precipitation tests with the fraction of another Vi-containing strain, Ty 58, are shown in Table IV. The upper positive fraction was almost inactive, so that the alpha component, which constituted almost all of the material in this fraction, can be said to have had at most very little activity. The lower positive fraction, which contained only a trace of gamma, was extremely active, and its activity may be ascribed to the content of beta component. In like fashion, the

TABLE V

Precipitin Activity of Fractions of Ty 2 Concentrates

	Precipitation				Turbidity				Concentration* in the undiluted fraction mg./ml.		
									Alpha	*Beta*	*Gamma*
Fraction	0.2	0.1	0.05	0.025	0.2	0.1	0.05	0.025			
Saline. .	0.1	0.2	0.25	0.275	0.1	0.2	0.25	0.275			
Antiserum	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1			
Upper positive	+	−	−	−	18	11	5	0	0.37	0.14	0
Lower positive..	++	++	+	−	31	25	23	16	0.41	1.26	0.30
Bottom Section	++	++	++	+	31	32	26	18	0.42	1.29	1.07
Upper Negative.....	++	−	−	−	24	17	11	6	0	0	0.75

* Calculated from the areas of the electrophoretic diagrams and the final positions of the electrophoretic boundaries on the assumption that the specific refractive increment of each constituent was 0.0018.

activity of the upper negative fraction was due to the content of gamma component.

The fractions of strain Ty 58 were, then, similar to those of strain Ty 2, inasmuch as both the beta and the gamma components were serologically reactive, while the alpha component was not. Although both strains showing activity in both beta and gamma fractions contained Vi antigen, this double activity cannot be considered characteristic until more strains have been studied.

Combiesco and Soru (12) subjected the trichloroacetic acid extracts of typhoid bacilli to electrophoresis in a U-tube and by precipitin reactions

determined the concentrations of the antigens at the poles after about sixty minutes. They concluded that the Vi antigen carried a negative charge four to five times as great as that on the O antigen. However, extracts from a strain containing both Vi and O antigens migrated at the same rate as the pure Vi antigen.

It is difficult to compare our results directly with theirs since we used the chemically-untreated concentrates of the culture fluid and a refined method of electrophoresis, while Combiesco and Soru used trichloroacetic acid extracts of the whole bacilli and a less exact electrophoretic technique. We may note, however, that while both the beta and gamma constituents were serologically reactive in the Ty 2 strain (which, like the strain used by Combiesco and Soru, contains both the Vi and the O antigens), the reactivity of the beta component exceeded that of the gamma component towards antisera to both the O and Ty 2 strains (Table IV).

Summary

Concentrated solutions of the growth products of four strains of typhoid organisms, namely H, which is motile; O which is non-motile; and Ty 2 and Ty 58, both of which contain Vi antigen, were examined by electrophoresis in the Tiselius apparatus. Each concentrate contained three constituents, alpha, beta and gamma, in the order of decreasing mobility. The corresponding constituents of the four concentrates were similar but not identical in mobility, and were present in varying proportions.

Each component of the four strains conferred some protection on mice against infection by the virulent typhoid organism Ty 63. The components of the Ty 2 and Ty 58 strains were more protective than those of the H and O strains, but in any strain no one constituent far exceeded either of the others in protective action; the effectiveness of the individual components in most cases paralleled that of the unfractionated concentrate.

Serological tests on the fractions separated by electrophoresis demonstrated that in the H and O strains serological reactivity was restricted to the gamma component. In the Ty 2 and Ty 58 strains, on the other hand, which contain the Vi antigen, the beta component was found to be serologically active also; and in the Ty 2 strain, at least, this component appeared to be the one with which the Vi antigen was associated.

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Crystalline Urease vs. Urease Preparations for Urea Determinations

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Introduction

Despite the fact that urease was obtained in crystalline form 18 years ago (1) and that it is probably easier to prepare and to recrystallize than any other enzyme, nevertheless, the crystalline product has not been employed generally for analytical work. That this is the case is made evident through the appearance, from time to time, of articles which give directions for the removal of interfering impurities from commercial preparations of urease (2). Two misconceptions about crystalline urease appear to exist. The first is that it can be prepared only by a person who possesses unusual skill. This is untrue. Any person who can prepare photographic developer should have no difficulty with urease. The second misconception is that some special sort of jack bean meal is needed. This also is untrue. For about 15 years the Arlington Chemical Company of Yonkers, N. Y., has been selling jack bean meal from which a satisfactory crop of urease crystals can be obtained. It is our experience that concentrated solutions of crystalline urease remain servicable for analytical work for many months, provided they are kept in an ice chest at from 3 to 6°C. The few precautions which must be observed apply to both crystalline urease and to impure urease. For example, one must employ water distilled from glass and one must avoid the use of glassware which has a film of mercury, silver, or copper on its inside surface.

In this laboratory Howell (3) has already advocated using crystalline urease in the determination of blood urea. He has shown that upon standing with water both jack bean meal and certain commercial urease preparations produce ammonia, whereby erroneous values are likely to be obtained. Howell was able to avoid ammonia production through the

employment of 5 to 20-hour old jack bean meal extracts, together with the use of citrate buffer, which maintained the digests at pH 6.0 to 6.6.

Extensive use has been and is being made of urease prepared jack bean meal by the method of Van Slyke and Cullen (4). Here, urease is extracted from the meal with water, followed by precipitation of the centrifuged extract with a large excess of acetone and drying in vacuum. We do not consider this procedure to be advisable. The extraction of the urease achieves a certain concentration, it is true, since the insoluble matter is left behind, but when the extract is poured into acetone the urease is partly inactivated. The net result is a product only about 3-fold more active than the original meal. Moreover, the product is likely to produce a comparatively large quantity of ammonia when it stands with water.

Archibald and Hamilton (2) have reported that jack bean urease preparations, made according to the procedure of Van Slyke and Cullen, contain about 80 mg. of canavanine per gram. They could remove this amino acid by dialysis. We have found that crystalline urease contains a small amount of dialyzable nitrogenous material which gives a ninhydrin test. This is probably canavanine. In one of our experiments the crude crystalline urease from 200 g. of "Arlco" jack bean meal contained 87.5 mg. of protein (N $\times$ 6.25). Upon dialyzing overnight it lost 1.24 mg. of nitrogen. This would correspond to nearly 4 mg. of canavanine. A solution of recrystallized urease contained 21.4 mg. of protein and lost 0.147 mg. of nitrogen by overnight dialysis. This would correspond to 0.46 mg. of canavanine. Crude crystalline urease contains traces of a largely inactive arginase (5). This becomes active if a cobaltous or manganous salt is added. Hence, there is a possibility that if one should add cobalt or manganese ions to a solution of crude crystalline urease the activated arginase would form urea from the canavanine and this urea would be decomposed immediately to form ammonium carbonate. However, crystalline urease is so active that one needs to employ only 0.007 mg. for each blood urea analysis. The quantity of ammonia which could be produced from the canavanine in this amount of urease could not be detected by ordinary means.

Both the arginase and the canavanine in urease can be decreased by recrystallizing. Although we consider the recrystallization of urease that is to be employed for urea determinations to be entirely unnecessary, we wish to call attention to the recrystallization method of A. L. Dounce (6). This method is rapid and simple and is an advance over the original

method of Sumner (7). We wish to point out, however, that there is an error in Dounce's article. He stated that 0.5 M citrate buffer of pH 6.0 was prepared by mixing 9 volumes of 0.5 M disodium citrate with 1 volume of 0.5 M citric acid. This should be changed to: "95 volumes of 0.5 M trisodium citrate with 5 volumes of 0.5 M citric acid."

No doubt many analysts have little or no idea how much crystalline urease it is necessary to employ for the analysis of urea in the Folin-Wu blood filtrate. The following experiment will furnish the needed information:

We dissolved the urease crystals obtained from 200 g. of "Arlco" jack bean meal by the Sumner procedure (1) in 13 ml. of water. One ml. of this solution was mixed with 50 ml. of 2 per cent gum arabic of pH 6.0. One drop of this gum arabic-urease solution (17.5 drops per ml.) was found to be ample to decompose completely 0.5 mg. of urea in 10 minutes at 42°C. when the total volume of the digest was 5 ml. and 5 drops of 0.5 M citrate buffer of pH ca. 6.0 had been added to keep the digest from becoming alkaline. *It can be calculated, therefore, that the urease crystals from 200 g. of "Arlco" jack bean meal will suffice for 11,500 blood urea analyses.*

For the convenience of the reader we repeat directions for the preparation of crystalline urease: Weigh 100 g. "Arlco" jack bean meal into a 1 liter beaker. Using glass-distilled water and acetone redistilled from fuzed calcium chloride and soda lime, prepare 500 cc. of 32 per cent acetone at 22 to 28° C. Pour this diluted acetone upon the meal and, as rapidly as possible, stir the meal into a suspension with a clean wooden stick. Pour the material upon a 32 cm. Whatman No. 1 filter. When 50 to 100 ml. of filtrate have come through place the filtering material in an ice chest at 3 to 6°C. and allow to remain overnight. Preferably using 250 ml. centrifuge bottles, centrifuge the filtrate in a cold room. Carefully decant the supernatant liquid and drain the tubes without agitating the sediment of urease crystals. Now dissolve the urease crystals and transfer the solution to a 15 ml. centrifuge tube, using 2 ml. at a time, a total of 6 ml. of water. Mix the turbid suspension and centrifuge it at high speed from 1 to 2 hours, or until the liquid is nearly clear. Pipette off the urease solution. Keep this in the ice chest in a stoppered pyrex test tube. It may be advisable to add a drop of toluene from time to time, although we have not found this necessary. A working solution of urease is prepared by mixing 0.1 ml. of the concentrated solution with 10 ml. of 2 per cent gum arabic which has been brought to about pH 6.0.

The analyst may wonder how to tell whether this dilute urease solution is still potent after the elapse of several weeks. We think that the simplest method is the following. One keeps on hand well preserved with toluene a solution containing 0.5 mg. of urea in 5 ml. One adds 1 drop of the urease to 5 ml. of urea solution and then 5 drops of the 0.5 M citrate buffer of pH 6.0. The test is placed in water at

40 to 45°C. and left for 10 minutes. The digest is then transferred to a 200 ml. volumetric flask, diluted to two-thirds volume, Nesslerized with 20 cc. of Nessler solution, diluted to the mark, mixed and read in the photoelectric colorimeter. Consultation with the standard curve should show 0.233 mg. of nitrogen. If less nitrogen than this is present the urease should be discarded.

Summary

The very considerable advantages of using crystalline urease for the determination of blood urea have been pointed out. The method of preparing crystalline urease is again described.

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The Isolation of Nicotinic Acid from Milk and its Role as an Essential Growth Factor for Acetobacter Suboxydans

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Introduction

The existence of a factor other than biotin, which is essential for the growth of the organism *Acetobacter suboxydans* was demonstrated by Underkofler, Bantz, and Peterson (1) in biotin concentrates prepared from milk. Although experiments in this laboratory indicated that crystalline nicotinic acid or its amide possesses the activity of the essential factor it was thought advisable to isolate the active principle and confirm its identity since nicotinic acid had not been previously isolated from milk.

Nicotinic acid was first isolated from rice bran (2, 3, 4) and later from yeast (5) and more recently identified as the anti-black tongue factor (6) with the isolation of the amide from liver. The free acid was also isolated from yeast (7) and liver (8, 9) and shown to be a necessary growth factor for *Corynebacterium diphtheriae* (7) and *Staphylococcus aureus* (9).

A biotin concentrate from cow's milk which assayed from 0.3% to 0.5% of nicotinic acid by the method described in this paper, was esterified with acidic methanol, the crude ester dissolved in chloroform and then passed through a column of Decalso according to the directions given for the purification of biotin (10). After concentration of the chloroform eluate *in vacuo* a residue representing a ninefold purification was obtained. The active principle was extracted from the distillate with dilute hydrochloric acid resulting in a thirteen-fold purification. The aqueous

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acid extracts yielded a crystalline precipitate upon treatment with potassium mercuric chloride solution. Nicotinic acid was obtained from this compound by successive treatments with sodium hydroxide, copper acetate and hydrogen sulfide.

The assay procedure used to determine the activity of our concentrates was based on the growth response of *A. suboxydans* to nicotinic acid in the simplified basal medium of Underkofler, Bantz, and Peterson (1). Since the growth was proportional to the amount of nicotinic acid present, the nicotinic acid content of the sample was calculated by comparison with the growth obtained in a standard. The accurate portion of the standard curve ranged from 0.25 to 3.0 γ of nicotinic acid. Nicotinic acid and the amide were equally effective in stimulating the growth of *A. suboxydans*.

EXPERIMENTAL

A. Assay Procedure

Basal Medium. The basal medium contained the following ingredients per 100 ml.: 10 g. glycerol, 0.6 g. hydrolyzed casein, 20 mg. tryptophan, 15 mg. cystine, 100 mg. K_2HPO_4, 100 mg. KH_2PO_4, 40 mg. $MgSO_4 \cdot 7H_2O$, 2 mg. NaCl, 2 mg. $FeSO_4 \cdot 7H_2O$, 2 mg. $MnSO_4 \cdot 3H_2O$, 200 γ calcium pantothenate and 2 γ *p*-aminobenzoic acid. The pH of the medium was adjusted to 6.0.

Preparation of Inoculum. Stock cultures of the test organism, *A. suboxydans* (A.T.C.C. # 621) were maintained on wort agar slants. Cultures for inocula were grown in glycerol-yeast extract broth (glycerol 5.0%; Difco yeast extract 0.5%; pH 6.0) and transferred daily from preceding broth cultures. A return to the stock culture was made at weekly intervals. A 48-hour culture in 10 cc. of broth was centrifuged and washed twice with sterile 0.85% sodium chloride solution. The washed cells were resuspended in 15 cc. of the saline solution. One drop of this suspension per flask served as the inoculum.

Assay. Five cc. of the basal medium and dilutions of the sample made up to 5 cc. with distilled water were placed in 50 cc. Erlenmeyer flasks. Similarly, standard flasks were prepared containing from 10 to 0.05 μg. of nicotinic acid (or nicotinamide). Blanks containing no nicotinic acid were included. The flasks were plugged with cotton, autoclaved for 15 minutes at 15 pounds pressure, inoculated and incubated at 30°C. for 48 hours. After incubation, 11 cc. of water were added to each flask and the contents mixed thoroughly. The amount of growth was determined by measuring the turbidity in a photoelectric nephelometer. Graphs were constructed by plotting the nephelometer readings against the logarithms of the weights of the sample and standard. Comparison of the sample and standard was made at the half maximum point. The nicotinic acid content of the sample was calculated from these comparison values (see Fig. 1 and Table I).

B. Isolation and Identification

Isolation of Methyl Nicotinate Mercuric Chloride. Fifty grams of a biotin concentrate containing 180 mg. of nicotinic acid, as determined by the above assay procedure, were esterified by refluxing for one hour with anhydrous methanol containing 5% of dry hydrogen chloride. The solvent was removed *in vacuo* and the residue dissolved in 100 cc. of water, neutralized with solid sodium bicarbonate and extracted three times with

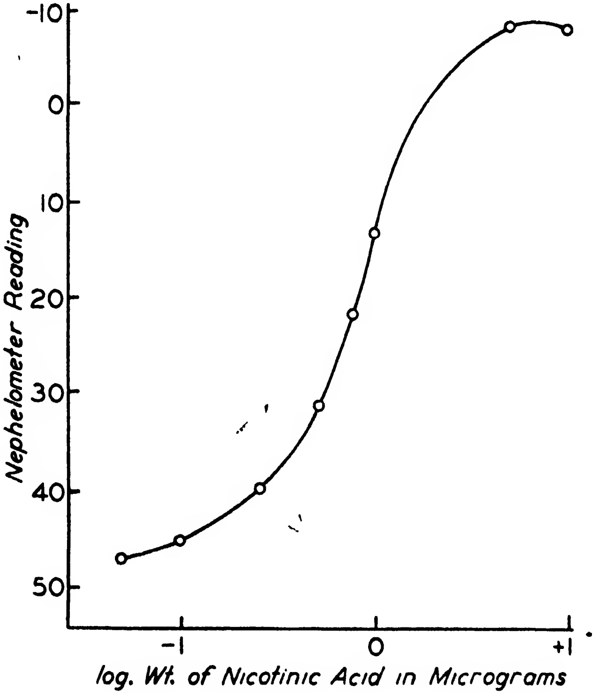

FIG. 1

100 cc. portions of ethyl acetate. The ethyl acetate extracts were dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* and redissolved in chloroform. The chloroform solution was further purified by passing it through a column (4′x1″) of Decalso. After collection of the chloroform eluate, the solvent was removed by distillation and the residue distilled at 0.1 mm. pressure. Four grams of distillate (Fraction D, Table I) assaying 152 mg. of nicotinic acid was obtained. This was extracted three times with 15 cc. portions of 2% hydrochloric acid and the

aqueous extracts (Fraction E, Table I) adjusted to pH 6.5 with sodium hydroxide and treated with 5 cc. of a 10% solution of potassium mercuric chloride. After standing a short time white needles separated which were collected by filtration and recrystallized from methanol. One half gram of methyl nicotinate mercuric chloride compound was obtained which is a 62.5% yield based on the biotin concentrate. It melted at 189–190°C. (sealed tube).

$C_{14}H_{14}O_4N_2HgCl_2$. Calculated: N = 5.13. Found: N = 5.18.

Synthesis of Methyl Nicotinate Mercuric Chloride. Nicotinic acid (123 mg.) was refluxed one hour with 25 cc. of methanol containing 5% of concentrated sulfuric acid. The excess solvent was removed *in vacuo* and the residue dissolved in 10 cc. of water and enough sodium bicarbonate added to neutralize the acid. The solution was extracted thrice with 10 cc. portions of ethyl acetate. The extracts were dried over sodium sulfate and the solvent removed *in vacuo*. The residue was taken up in 25 cc. of water and 5 cc. of a 10% potassium mercuric chloride solution was added. Characteristic needles formed slowly and after recrystallization from methanol melted at 189–190° (sealed tube). A mixed melting point with the isolated mercuric chloride compound showed no depression.

TABLE I

Nicotinic Acid as Growth Factor for A. Suboxydans

Sample	Micrograms giving half maximum growth	Nicotinic acid *per cent*
Nicotinic acid	0.55	100.0
Fraction E	1.20	45.8
Fraction D	15.1	3.64
Biotin #1000*	132.0	0.41

* Biotin Concentrate #1000, Research Laboratories, S.M.A. Corp.

Isolation of Nicotinic acid. To 110 mg. of isolated methyl nicotinate mercuric chloride compound were added 4 cc. of 10% sodium hydroxide. A yellow amorphous precipitate of mercuric oxide formed which was removed by filtration through filter-cel. The filtrate was refluxed one hour and partially neutralized with hydrochloric acid. A small amount of flocculent precipitate was removed as before and the filtrate exactly neutralized and treated with a copper acetate solution until no more precipitate appeared. The copper acetate was collected by filtration,

suspended in 2 cc. of water and decomposed by passing in hydrogen sulfide. The copper sulfide was removed by filtration through filter-cel and the filtrate concentrated *in vacuo* to dryness, yielding 20 mg. (59%) of white crystalline compound. This material was sublimed at 0.1 mm. (100–110°C. oil bath temp.) and the sublimate melted at 232–234°C. (sealed tube). A mixture with an authentic sample of nicotinic acid showed no depression in melting point. The picrate of the isolated nicotinic acid was prepared and melted at 220–221°C. A mixed m.p. with synthetic nicotinic acid picrate showed no lowering.

Summary

1. Nicotinic acid has been isolated from a milk concentrate.

2. A method of assay for nicotinic acid is described.

3. Nicotinic acid has been shown to be an essential growth factor for *A. suboxydans*.

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Relationship of Iron Nutrition to the Synthesis of Vitamins by Torulopsis utilis

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Introduction

Certain similarities in the physiological actions of essential trace elements and organic growth factors of the vitamin B group (for example, the participation by members of both groups of nutritional factors in essential enzymic systems) suggest the possibility that variations in the adequacy of trace-element nutrition may influence the extent of vitamin synthesis by microorganisms. Aside from the fundamental biochemical significance of such interrelations, the problem is of interest because of recent commercial development of vitamin-bearing products of microbial origin. In this Laboratory attention has been centered on the production of yeast high in vitamin content from the press juices of waste and cull fruits.

Evidence that inorganic nutritional conditions affect the synthesis of organic growth factors is available. Riboflavin has attracted particular attention, partly because of its pigmented nature. Thus Lavollay and Laborey (1941) found that the initiation of substantial riboflavin synthesis by certain strains of *Aspergillus niger* is directly associated with magnesium deficiency. Knobloch and Sellmann (1941) later showed that riboflavin production by many strains of *Aspergillus niger* is affected by the inorganic nutrition, although wide strain differences were observed. Markedly increased yields of riboflavin can be obtained from *Clostridium acetobutylicum* by additions of calcium carbonate (Yamasaki, 1941); this response may be due to certain effects on the inorganic

* This is one of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

nutrition of this bacterium. A number of other examples, for other vitamins as well as for riboflavin, could readily be cited.

The iron nutrition of *Torulopsis utilis*, the "wild" yeast extensively used for fodder-yeast production in continental Europe and elsewhere, was chosen for a preliminary study of the relationships of trace-element nutrition to vitamin synthesis because it is comparatively easy to induce nutritional deficiency of iron and because the biochemical functions of iron are as well established as those of other trace elements. Use of recently developed microbiological vitamin-assay methods permits the rapid completion of experiments that permit statistical evaluation. The following study deals with the effects of variations in the iron content of the nutrient solution from deficiency to inhibitory excess on the synthesis of ten individual growth factors of the vitamin B complex by *Torulopsis utilis.*

Experimental

Microorganism

Henrici (1941) has pointed out that the yeast commonly called *Torula utilis* in literature with industrial implications is correctly called *Torulopsis utilis.*[1] This yeast is able to grow on a wide variety of natural and synthetic substrates with yields frequently exceeding 50 g. of dry yeast per 100 g. of fermentable material. Such yields depend on adequate aeration of the cultures and on the incremental addition of nutrients. In the present study maximum yields of 25 to 35 g. of dry yeast were obtained per 100 g. of sucrose under aeration without supplemental feeding after inoculation.

Torulopsis utilis is autotrophic with respect to vitamins and thus possesses well developed vitamin synthesizing capacities. Its vitamin content, when grown on various media, appears to resemble that of unfortified bakers' or brewers' yeasts fairly closely. Vitamin assays for this study were performed on the whole cultures instead of on cells and culture liquors separately. The assays thus represent the total synthesis of the vitamins with the exception of that portion that is destroyed or metabolized, and thus for certain vitamins the assays are considerably higher than the normal contents for this yeast. The question of the extent of retention of synthesized vitamins within the yeast cells is not considered in these experiments. Previous studies with this yeast

[1] The strain used in this study is carried by the Stock Culture Section of the Northern Regional Research Laboratory, Peoria, Illinois as No. Y-900.

(Lewis, Stubbs, and Noble, 1944) grown on prune wort, with incremental addition of nutrients and aeration, indicated that about half of the riboflavin, nine-tenths of the nicotinic acid, two-thirds of the pantothenic acid, practically all of the biotin, and a fourth of the *p*-aminobenzoic acid synthesized was retained by yeast harvested immediately following the most active propagation period. Further vitamin excretion upon standing in contact with culture medium for 18 hours in the refrigerator was apparent only in the case of *p*-aminobenzoic acid, at which time only 16 per cent of the total synthesized remained in the yeast. In cultures refrigerated for long periods without separation of the yeast, as little as 4 per cent of the total *p*-aminobenzoic acid was retained in the yeast.

Cultural Conditions

The yeast cultures were grown at 30°C. in small Pyrex cylinders fitted with fritted-glass, false bottoms, and cotton-filled tubes for filtering air. The culture vessels were soaked overnight in 3 *N* HCl to remove traces of iron. After thorough rinsing, 200-ml. aliquots of sucrose-salts nutrient solution[2] were added and the entire assemblies were sterilized for 15 minutes in steam under 10 lbs. of pressure. After cooling, 1-ml. aliquots of sterile solutions of $FeSO_4 \cdot 7H_2O$ in concentrations required to give six rates of added iron varying from 0 to 10 p.p.m., were added to all flasks. A slight turbidity was formed on the addition of 10 p.p.m. of Fe and a barely perceptible turbidity on the addition of 3 p.p.m.

The flasks were then inoculated with 2 ml. (about 1 per cent by volume) of a 24-hour yeast culture grown in the same manner without added iron. This inoculum supplied approximately 0.3 mg. of dry yeast per flask. The cultures were aerated, slowly at first and more vigorously after growth was well started. Cessation of growth appeared to be accompanied by increased formation of foam. No lard or other anti-foaming agent was added. After 48 hours of aeration, growth had

[2] Nutrient solution (adjusted with KOH to give pH 6.0 after sterilization and addition of iron treatments; reagent salts used throughout):

	per cent		*p.p.m.*
Sucrose	2	Zn (sulfate)	0.30
NH_4NO_3	0.20	Mn (sulfate)	0.075
KH_2PO_4	0.035	Cu (sulfate)	0.075
$MgSO_4 \cdot 7H_2O$	0.025	Na (sulfate)	0.058
		Mo (MoO_3)	0.020

ceased, and the whole cultures were transferred to sample bottles and stored at 0°C. until vitamin assays could be performed.

The sucrose was almost completely utilized in cultures containing added iron and approximately half-utilized in the iron-deficient cultures.

Growth was measured turbidimetrically during incubation. After harvesting, yeast yield was determined gravimetrically after centrifugation, washing, and drying; this measurement was reproducible within 1 to 2 per cent. Vitamin-synthesis rates were calculated on the basis of the dry yeast determination.

Vitamin Assay Methods

Three types of microbiological assays were used: (1) a yeast-fermentation method (thiamin); (2) turbidimetric yeast growth methods using *Saccharomyces carlsbergensis*; and (3) acidimetric Lactobacillus growth methods. Crystalline vitamins served as standards in all cases except that of Norit-eluate factor isotels.[3] The Norit eluate factor assays were standardized against a folic acid concentrate[4] having a potency of 3100; it was 7.7 per cent as active as standard material having a potency of 40,000 (Mitchell, Snell, and Williams, 1941; Mitchell and Snell, 1941). The thiamin assays are expressed as thiamin chloride hydrochloride, pantothenic acid as calcium pantothenate, biotin as the free acid, pyridoxin as the hydrochloride.

The reliability of several of the methods has been extensively investigated in various laboratories; in other cases the methods are new and relatively untested. Numerous experiments on the applicability of microbiological assays to *Torulopsis utilis* have been made in this laboratory, but they need not be outlined here. The results are assumed to be reasonably reliable within the limitations cited in individual cases.

Thiamin Assay Thiamin was determined by the micro-fermentation method of Atkin, Schultz, and Frey (1939). The method measures certain isotels of thiamin as well as true thiamin. It is made specific for thiamin by measuring the fermentation-stimulating activity before and after sulfite destruction of thiamin, a treatment which does not affect the activity of hydrolytic products of thiamin that behave similarly to thiamin in the fermentation test (Schultz, Atkin, and Frey, 1942). The yeast cultures were prepared for assay by heating for 30 minutes

[3] Compounds related by their common ability to perform the same function are called "isotels" (Williams, 1943).

[4] This folic acid concentrate was very generously supplied by Dr. R. J. Williams of the University of Texas.

in flowing steam. Assays were made both for total thiamin isotels and for true thiamin by means of the sulfite technique. For reasons to be discussed below, the author believes that total fermentation stimulation is a better measure of thiamin synthesis than is the determination of residual true thiamin.

Yeast Growth Methods. Pyridoxin was determined by the method of Atkin, Schultz, Williams, and Frey (1943), using *Saccharomyces carlsbergensis*. Pyridoxin was liberated by acid autoclaving as recommended by these authors.

Inositol was determined by a modification of the pyridoxin assay consisting principally in the replacement of inositol in the basal medium of Atkin and colleagues by 0.10 p.p.m. of pyridoxin. The most satisfactory assay range lies between 3 and 12γ of inositol per tube. Samples were prepared by autoclaving the centrifuged cells for 30 minutes in 1 *N* H_2SO_4, followed by recombination with the culture liquor and neutralization. Wide variability between similarly prepared tubes necessitated considerable replication, yet the assay values appear to be reliable.

Lactobacillus Assays. Lactobacillus assay methods, which depend on the relation between vitamin deficiency and lactic acid production by *Lactobacillus casei*[5] and *Lactobacillus arabinosus* 17-5,[6] are applicable for a considerable number of the B group of vitamins. The general technique is common to all of these methods. Acid production was determined by titration after three days of incubation at 37°C. for methods using *Lactobacillus casei*, and at 30°C. for methods using *Lactobacillus arabinosus*.

It was found convenient to formulate a general vitamin supplement from which the particular factor being assayed could be omitted. The use of such a formula permits elimination of the treated yeast extract supplements prescribed in the riboflavin and pantothenic acid assay methods. The formula allowed 0.5 p.p.m. of thiamin hydrochloride, 0.2 p.p.m. of riboflavin, 0.5 p.p.m. of nicotinic acid, 0.1 p.p.m. of calcium pantothenate, 0.5 p.p.m. of pyridoxin hydrochloride, and 0.01 p.p.m. of *p*-aminobenzoic acid to the medium. In addition, 0.0004 p.p.m. of biotin (free acid) was supplied in the nicotinic acid, pyridoxin, Norit eluate factor, and *p*-aminobenzoic acid assays. For pyridoxin isotels assays, 0.001 p.p.m. of folic acid in the form of a concentrate having a potency of 3100 was also added.

HCl-hydrolyzed Labco vitamin-free casein was used in all cases. It was treated with Norit A (Pfanstiehl) at pH 3 when necessary to remove small amounts of the vitamins being assayed. This treatment was unnecessary for pantothenic acid assays. A single Norit treatment was used for nicotinic acid and pyridoxin isotels assays; a double Norit treatment in the other cases.

All assay cultures except those for pyridoxin isotels were grown in Pyrex test tubes (18 × 150 mm.). The reproducibility of the pyridoxin isotels assay cultures was greatly enhanced by growing 10-ml. cultures in 2-oz. softglass bottles which provided shallow layers having about 1.3 square inches of exposed surface, un-

[5] American Type Culture Collection, Georgetown University Medical School, Washington, D. C., No. 7469.

[6] American Type Culture Collection, Georgetown University Medical School, Washington, D. C., No. 8014.

doubtedly because the pyridoxin requirement of *Lactobacillus casei* is a function of the oxygen supply (Bohonos, Hutchings, and Peterson, 1942).

In most cases the yeast cultures were prepared for assay by treating the centrifuged yeast cells to liberate bound vitamins, and recombining the treated cells with the culture filtrates. This treatment usually consisted of liquefaction of the yeast with ethyl acetate followed by at least 24 hours of autolysis at room temperature. Biotin was liberated by hydrolysis in 3 N HCl for 30 minutes under 15 pounds of steam pressure. Alkaline hydrolysis, which gives greatly enhanced *p*-aminobenzoic acid assays with many materials, gave small or no increases in assay values for *Torulopsis utilis* cultures.

References to the Lactobacillus methods used, except for the modifications noted above, are as follows: riboflavin, Snell and Strong (1939); nicotinic acid and biotin, Snell and Wright (1941); pantothenic acid, Pennington, Snell, and Williams (1940); pyridoxin isotels and Norit eluate factor isotels (folic acid), Landy and Dicken (1942); and *p*-aminobenzoic acid, Lewis (1942).

Results and Discussion

The effects of iron additions to an iron-deficient medium on the growth of *Torulopsis utilis* are presented in Table I. It is apparent that the basal medium was sufficiently deficient in iron to reduce the yield of dry yeast to approximately 20 per cent of the normal yield. This deficiency was largely overcome by the addition of 0.1 p.p.m. of iron. The optimum was not reached until 3 p.p.m. of iron was added; 10 p.p.m. of iron gave decreased yields. The inhibiting effects of the higher concentrations of iron are more clearly expressed in growth rate as indicated by turbidity than in final yield. It may be noted that in the case of the 23-hour readings, the optimal iron additions were 0.1 and 0.3 p.p.m., higher concentrations being somewhat inhibitory.

The pigmentation of the cells varied from an opaque white for the iron-deficient cultures to a light tan color for cultures containing added iron. Maximum pigmentation was observed at 0.3 to 3 p.p.m. of iron. Lack of pigmentation in several species of bacteria grown on media biologically deficient in iron was noted by Waring and Werkman (1943). Elvehjem (1931) reported pale color and deficiency of cytochrome in iron-deficient bakers' yeast.

Thiamin results are presented in Table I. Determination of total thiamin isotels (from total fermentation-stimulating activity) is assumed to be the better measure of total thiamin synthesis than is determination of true thiamin, since in the former case certain hydrolytic and metabolized products of thiamin are measured together with true thiamin. The true thiamin was also assayed by the sulfite technique and the percent of true thiamin in the total thiamin isotels of these cultures is

noted in Table I. By inspection of the turbidity measurements it may be seen that the iron-deficient cultures attained their maximum growth before cultures containing intermediate concentrations of iron, while growth in the cultures containing high concentrations of iron lagged behind all of the others. It is thus clear that the iron-deficient cultures were aerated for appreciably longer periods after maximum growth was attained than any of the other cultures. This undoubtedly is the

TABLE I

Effect of Added Iron on Growth and Thiamin Synthesis by Torulopsis utilis

	Growth			Thiamin		
Added Iron	Turbidity at 23 hrs.	Turbidity at 48 hrs.	Dry Yeast at 48 hrs.	Culture Basis*	Dry Yeast Basis*	Remaining as True Thiamin
p.p.m.	*Klett units*†	*Klett units*†	*mg./ml.*	*γ/ml.*	*γ/g.*	*per cent*
0.00	210 ± 20‡	350 ± 60	1.16 ± 0.17	0.124	106 ± 5	11 ± 6
0.1	540 ± 90	1190 ± 160	4.20 ± 0.53	0.192	46 ± 5	63 ± 8
0.3	450 ± 20	1410 ± 40	4.83 ± 0.13	0.225	47 ± 2	65 ± 4
1	180 ± 40	1320 ± 30	4.76 ± 0.24	0.218	46 ± 2	75 ± 4
3	70 ± 40	1720 ± 170	6.04 ± 0.65	0.212	35 ± 1	73 ± 5
10	240 ± 40§	1230 ± 150	4.14 ± 0.56	0.173	43 ± 5	82 ± 4

* Total fermentation-stimulating activity, expressed as thiamin hydrochloride.

† The number of Klett units of turbidity is obtained by multiplying the scale reading on the Klett-Summerson photoelectric colorimeter (660 mμ filter) by the dilution factor used to reduce the turbidity to a measurable level. Calculations based on the 48-hour determinations give an average conversion factor of 289 ± 11 (s.d.) Klett units per mg. of dry yeast per milliliter of culture.

‡ The data in this and following tables consist of means and standard errors for determinations on three cultures for each level of iron.

§ The reason for the reversal of growth trend at 23 hours for 10 p.p.m. of added iron is not apparent. The Klett value of the slightly turbid uninoculated medium approximated 7 units.

explanation for the low proportion of true thiamin to total thiamin isotels in these cultures, it being recognized that thiamin is destroyed by aerated nonproliferating yeast (Van Lanen, Broquist, Johnson, Baldwin, and Peterson, 1942). If this interpretation is accepted, it is clear that iron deficiency results in a marked increase in the rate of thiamin synthesis, as indicated by an average of about 106 γ per g. of dry yeast for cultures lacking added iron compared with an average of about 35 γ per g. of yeast grown in the presence of 3 p.p.m. of iron.

Riboflavin results are presented in Table II. The differences are not large, but it may be noted that cultures lacking added iron and cultures containing high concentrations of iron synthesized about 40 per cent more riboflavin per unit of dry yeast than did the cultures containing 0.1 p.p.m. of added iron. The increased riboflavin synthesis in the case of iron deficiency was not unexpected since it had been previously reported that cyanide (which blocks iron-containing respiratory mechanisms) results in an increased flavin synthesis by brewers', bakers', and other yeasts (Pett, 1935, 1936; Burkholder, 1943). Likewise, Yamasaki (1941) found that the addition of 2 p.p.m. of iron inhibited riboflavin formation by *Clostridium acetobutylicum* almost completely. Since growth was increased under his conditions by the addition of iron, it might seem that in this case riboflavin production was dependent upon partial iron deficiency.

TABLE II

Effect of Added Iron on Riboflavin, Nicotinic Acid, and Pantothenic Acid Synthesis by Torulopsis utilis

	Riboflavin*		Nicotinic Acid†		Pantothenic Acid‡	
Added Iron	Culture Basis	Dry Yeast Basis	Culture Basis	Dry Yeast Basis	Culture Basis	Dry Yeast Basis
p.p.m.	*γ/ml.*	*γ/g.*	*γ/ml.*	*γ/g.*	*γ/ml.*	*γ/g.*
0.00	0.14	121 ± 8	1.22	1060 ± 50	0.20	185 ± 30
0.1	0.32	77 ± 1	3.32	760 ± 50	1.01	225 ± 45
0.3	0.44	92 ± 2	3.18	660 ± 10	0.97	200 ± 10
1	0.46	97 ± 4	3.15	660 ± 10	0.91	190 ± 10
3	0.61	102 ± 5	3.97	650 ± 20	1.23	200 ± 15
10	0.48	116 ± 3	2.55	620 ± 10	0.64	155 ± 15

* *Lactobacillus casei* assay.

† *Lactobacillus arabinosus* assay.

‡ *Lactobacillus casei* assay; pantothenic acid as calcium pantothenate.

Nicotinic acid synthesis results are given in Table II. The normal synthesis of about 650 γ of nicotinic acid per g. of dry yeast is increased to about 1000 to 1200 γ per g. by iron deficiency.

Pantothenic acid synthesis results are also given in Table II. Although the range of synthesis varied from 140 to 300 γ of pantothenic acid per g. of dry yeast, no clear-cut correlation with the state of iron nutrition of the yeast is apparent. It is not certain to what extent destruction of pantothenic acid, as the cultures become acidic (pH 3.0 to 3.8), has masked the true pantothenic acid picture.

Biotin results are presented in Table III. (The biotin assays exhibited pronounced downward trends of assay values with increasing size of assay aliquot, which diminishes the absolute significance of the data but probably does not obscure the true relation of biotin synthesis to iron nutrition, since all assays were performed simultaneously.) It may be seen that both iron deficiency and iron excess limited biotin synthesis to about 25 to 50 per cent of the somewhat variable normal synthesis.

Pyridoxin was assayed with two different microorganisms. The results, which are summarized in Table III, show that the *Lactobacillus casei* assays are roughly 40 times as great as the *Saccharomyces carlsbergensis* assays. These assays probably reflect a response of *Lactobacillus casei* to certain highly active isotels of pyridoxin, such as pseudopyridoxin (Snell, Guirard, and Williams, 1942). The *Lactobacillus casei* test has been shown to give pyridoxin assays 30 to 40 times greater than are given by *Neurospora sitophila* (*pyridoxinless* mutant, Beadle and Tatum, 1941) (Stokes, Foster, and Woodward, 1943), while Williams (1943) states that pseudopyridoxin has high activity for *Lactobacillus casei*. Assays with *Saccharomyces carlsbergensis* give pyridoxin values consistent with results obtained by chemical and animal assay methods (Atkin, Schultz, Williams, and Frey, 1943) and may be presumed to represent true pyridoxin. The syntheses of true pyridoxin and of pyridoxin isotels show similar responses to the state of iron nutrition, restriction of iron approximately doubling the extent of the syntheses.

TABLE III

Effect of Added Iron on Biotin and Pyridoxin Synthesis by Torulopsis utilis

	Biotin*		Pyridoxin†		Pyridoxin Isotels‡	
Added Iron	Culture Basis	Dry Yeast Basis	Culture Basis	Dry-Yeast Basis	Culture Basis	Dry-Yeast Basis
p.p.m.	*γ/ml.*	*γ/g.*	*γ/ml.*	*γ/g.*	*γ/ml.*	*γ/g.*
0.00	0.00045	0.39 ± 0.02	0.22	187 ± 10	8.6	7400 ± 1100
0.1	0.0034	0.79 ± 0.11	0.95	228 ± 16	26.7	7200 ± 1000
0.3	0.0044	0.91 ± 0.06	0.77	158 ± 7	29.3	6100 ± 200
1	0.0056	1.14 ± 0.34	0.60	126 ± 7	22.6	4700 ± 100
3	0.0085	1.32 ± 0.45	0.60	100 ± 8	21.0	3500 ± 500
10	0.0021	0.49 ± 0.06	0.42	103 ± 6	13.5	3300 ± 300

* *Lactobacillus arabinosus* assay; biotin as free acid.

† *Saccharomyces carlsbergensis* assay; pyridoxin as pyridoxin hydrochloride.

‡ *Lactobacillus casei* assay. Results expressed as pyridoxin hydrochloride.

In this case the vitamin synthesis was enhanced by variations in levels of added iron that did not greatly reducé the level of yeast production. This, of course, is the type of response that might prove of practical significance. Probably only a limited reduction in yeast yields would be permissible commercially in the interest of enhanced vitamin content.

Inositol synthesis results are presented in Table IV. Although the synthesis values are somewhat variable, it is clear that severe iron deficiency results in a reduction of inositol synthesis to 25 to 50 per cent of normal, while an excess of iron results in erratic reductions.

TABLE IV

Effect of Added Iron on Inositol, p-Aminobenzoic Acid, and Norit Eluate Factor Isotels Synthesis by Torulopsis utilis

Added Iron	Inositol* Culture Basis	Inositol* Dry-Yeast Basis	*p*-Aminobenzoic Acid† Culture Basis	*p*-Aminobenzoic Acid† Dry-Yeast Basis	Norit Eluate Factor Isotels‡ Culture Basis	Norit Eluate Factor Isotels‡ Dry-Yeast Basis
p.p.m.	*γ/ml.*	*γ/g.*	*γ/ml.*	*γ/g.*	*γ/ml.*	*γ/g.*
0.00	0.7	600 ± 200	0.0036	3.2 ± 0.2	0.014	12.4 ± 0.8
0.1	10.5	2300 ± 700	0.225	52 ± 15	0.080	18.7 ± 0.9
0.3	10.4	2200 ± 200	0.185	38 ± 1	0.116	24.2 ± 2.4
1	7.8	1700 ± 200	0.295	62 ± 2	0.116	24.3 ± 1.1
3	15.8	2600 ± 200	0.220	38 ± 13	0.113	19.2 ± 3.7
10	6.6	1400 ± 700	0.280	69 ± 14	0.134	31.2 ± 3.6

* *Saccharomyces carlsbergensis* assay.

† *Lactobacillus arabinosus* assay.

‡ *Lactobacillus casei* assay. Results expressed as folic acid concentrate of potency 40,000.

The data on the synthesis of *p*-aminobenzoic acid are also presented in Table IV. This synthesis was very adversely affected by iron deficiency, the rate of synthesis being reduced from roughly 60 γ of *p*-aminobenzoic acid per g. of yeast for normal yeast to about 3 γ per g. of yeast for yeast suffering from severe iron deficiency. In view of the striking effects noted, one is tempted to speculate as to a rather direct participation of iron in the mechanism of synthesis of *p*-aminobenzoic acid.

The Norit eluate factor for *Lactobacillus casei* has been considered as identical with folic acid (Hutchings, Bohonos, and Peterson, 1941) and the use of this organism suggested for the assay of folic acid (Landy and Dicken, 1942). However, Keresztesy, Rickes, and Stokes (1943) have announced a new bacterial vitamin which will replace folic acid for

Streptococcus lactis R but not for *Lactobacillus casei*. Since *Streptococcus lactis* R is the organism advanced by Mitchell, Snell, and Williams (1941) for the assay of folic acid, it seems preferable for the present to refer to the results of *Lactobacillus casei* assays as Norit eluate factor isotels. It may be seen from Table IV that severe iron deficiency limited synthesis of Norit eluate factor isotels to about 50 per cent of that found for yeast adequately supplied with iron.

No particular consistency was noted in the type or extent of response of vitamin synthesis to iron deficiency or excess among the various B vitamins studied. This is not surprising, since the functional and synthesizing mechanisms undoubtedly differ widely for the various vitamins. Likewise, the participation of iron in the synthesizing mechanisms may be more or less indirect. The effect of the physiological age of the cultures at the time of harvest, which varied with the state of iron nutrition, on the true thiamin picture has already been discussed.

Summary

Of ten growth factors of the vitamin B complex studied, the synthesis of nine by *Torulopsis utilis* is significantly affected by the iron nutrition of this yeast. Iron deficiency is accompanied by increased rates of synthesis per gram of yeast for thiamin, riboflavin, nicotinic acid, pyridoxin and pyridoxin isotels, and by decreased rates of synthesis for biotin, inositol, *p*-aminobenzoic acid, and Norit eluate factor isotels. The yeast grown in the presence of a concentration of iron sufficient to give a limited repression of growth gave increased vitamin synthesis rates for riboflavin and Norit eluate factor isotels and decreased synthesis rates for biotin and inositol. No effects on the synthesis of pantothenic acid were demonstrated. The responses were of varying magnitudes, a very striking effect being observed with *p*-aminobenzoic acid. The synthesis of this factor amounted to approximately 3 γ per gram of iron-deficient yeast as compared with about 60 γ per gram for yeast adequately supplied with iron. It is concluded that studies of mineral nutrition offer considerable promise in a search for factors affecting the microbiological syntheses of vitamins.

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A Study of Biotin Sulfone

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Introduction

An investigation of the yeast-growth-promoting properties of characterized degradation products and other derivatives of biotin has been undertaken as these products became available. The yeast-growth-promoting activity of the diaminocarboxylic acid (DAC) derivative of biotin has been presented in a preliminary report (1). This compound possessed about 10 per cent the activity of biotin when compared at the level of a one-half maximum growth increase. Desthiobiotin (2) has recently been reported by Melville, Dittmer, Brown, and du Vigneaud (3) to have the same yeast-growth-promoting activity as biotin.

Observations have also been made on the behavior of these compounds toward avidin. The activity of DAC was found to be unaffected by avidin, whereas the activity of biotin and that of desthiobiotin are completely nullified by avidin. The difference in behavior of biotin and DAC with regard to avidin supported the earlier suggestion by Melville, Hofmann, and du Vigneaud (4) that the urea ring might be essential for the interaction between avidin and biotin. The inhibition of desthiobiotin by avidin indicates that the sulfur atom of the biotin molecule is apparently not necessary for the inhibiting effect of avidin.

In the present paper we wish to report on the action of biotin sulfone (5) on yeast growth, its action with avidin, and its effect on egg white injury in the rat.

Experimental

Assay Method

The method used for determining the yeast-growth-promoting properties of biotin sulfone was essentially that described for the determination of biotin by Snell, Eakin, and Williams (6).

Basal Medium. The basal medium was identical with that described by Snell, Eakin, and Williams (6).

Inoculum. 0.6 mg. moist yeast (S. Cerevisiae No. 139) was added per 100 cc. of basal medium. The yeast for inoculations was grown on wort agar slants for 24 hours at 30°C.

Assay Vessels. When the work was started, 50 cc. pyrex Erlenmeyer flasks were used. Varying quantities of a solution of the compound to be tested or of a solution of crystalline biotin for the standard curve were measured into sterile flasks. The volume in each flask was adjusted to 2 cc. and without further sterilization of the flasks, 10 cc. of yeast-inoculated medium were added. For convenience during the latter part of this study pyrex test tubes (20 × 150 mm.) were used for the assay vessels. The quantities of solution containing the growth stimulant were adjusted to 1 cc., and then 5 cc. of inoculated medium were added. The assay cultures were incubated without plugs for 16 hours at 30°C. The test tubes were slanted at an angle so that the solution was about 5 cm. from the lip. All tubes were inclined at the same angle in heavy wooden blocks prepared for that purpose[1].

Biotin Standard. Simultaneously with each test, a standard biotin-yeast-growth curve was obtained from a solution of crystalline biotin or its methyl ester. The standard curve (Fig. 1) usually consists of 14 tubes of the following amounts of biotin per 6 cc. of final volume: $(0, 0, 0.5, 1, 2, 3, 4, 5, 8, 10, 20, 30, 40, 300) \times 10^{-4}\ \gamma$. The greatest growth-increase obtained was arbitrarily taken as the maximum.

Growth Measurements. The amount of yeast growth manifested by turbidity was measured by a Klett-Summerson photoelectric colorimeter with a blue filter (Klett No. 42). The colorimeter reading was adjusted to zero against the basal medium. In all of our yeast-growth curves, growth is expressed as turbidity units of the colorimeter.

Method of Avidin Assay. The assay method for avidin in avidin preparations, published by Eakin, Snell, and Williams (7), measures total avidin, *i.e.*, the free avidin plus any avidin that is present in combination with biotin. All avidin and egg white preparations tested by us contained between 5 and 20 per cent of the avidin as avidin-biotin complex. To obtain a measure of free avidin activity of egg white and avidin preparations the following procedure was employed.

The sample to be assayed was accurately weighed and dissolved in enough 2 per cent ammonium sulfate to give a solution which contained approximately 0.005 unit of avidin per cc.; 1 cc. of this avidin solution was added to 1 cc. of a solution of

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[1] If the culture tubes were incubated in the vertical position maximum growth increase was about 65 per cent of the usual maximum. Increasing amounts of biotin above 0.0010 γ per 6 cc. did not produce increasing amounts of growth if the tubes were incubated in the vertical position.

crystalline biotin which contained 0.01 γ biotin per cc. After it was allowed to stand at room temperature for 30 minutes, the mixture was diluted with 3.0 cc. of sterile water. From this mixture, aliquots of 1.0, 0.8, 0.6, and 0.4 cc. were measured into assay culture tubes, diluted to a volume of 1.0 cc., and 5 cc. of yeast-inoculated medium were added to each of them. These cultures were incubated simultaneously with a series of tubes used to obtain a biotin standard growth curve. The yeast growth which was obtained in each tube was a measure of the amount of biotin which was not inhibited by the avidin added. The biotin values were obtained directly from the standard growth curve of biotin. The difference between the quan-

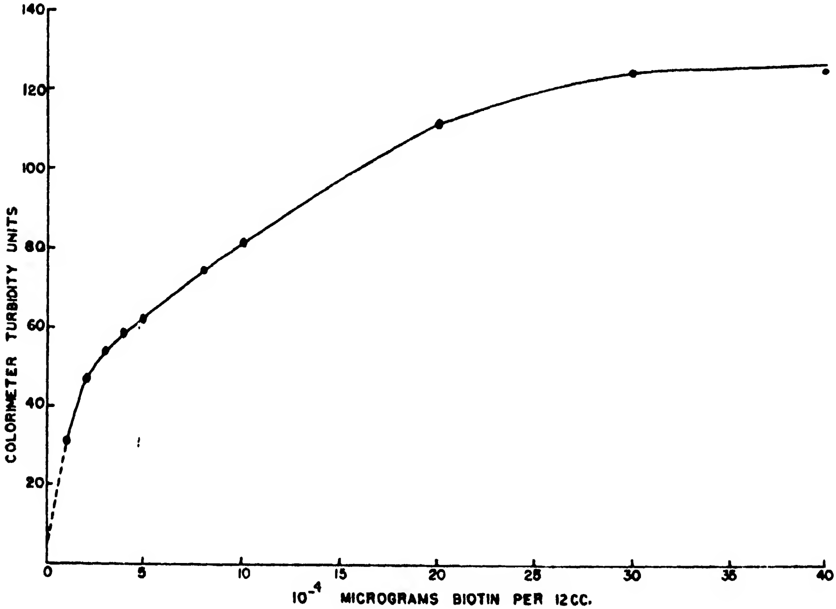

FIG. 1

A Typical Biotin Standard Yeast-Growth Curve

tities of biotin added and the amounts left for yeast growth is a measure of the active (or free) avidin. Table I shows the results of a typical assay where the sample tested was found to contain 4.2 units of avidin per gram.

Activity of Biotin Sulfone. When biotin sulfone in amounts varying from 0.02 to 0.20 γ in 2 cc. of distilled water was mixed with 10 cc. of yeast-inoculated medium, free of biotin, yeast growth was obtained which was proportional to the amounts of the compound added (Fig. 2). The yeast-growth response due to biotin sulfone reached a maximum at concentrations between 0.1 γ and 0.2 γ per 12 cc. It will be observed that

this maximum attained with the sulfone is much lower than the maximum growth obtained with biotin as the growth stimulant. If the typical growth curves (Figs. 1, 2) of biotin and biotin sulfone are compared, it will be noted that the "sulfone maximum" is reached at 50 to 55 colorimeter turbidity units whereas the "biotin maximum" is reached at 120–150 such units. Due to this low growth-maximum the activity of

TABLE I

Data of a Typical Avidin Assay

Tube No.	Biotin added	Avidin concentrate* added	Yeast growth	Biotin remaining	Biotin activity inhibited	Avidin activity
	γ	*mg.*	*Colorimeter units*	γ	γ	*units/g.*
1	0.0020	0.25	113	0.00092	0.00108	4.3
2	0.0016	0.20	104	0.00078	0.00082	4.1
3	0.0012	0.15	90	0.00057	0.00063	4.2
4	0.0008	0.10	77	0.00039	0.00041	4.1

* This sample of dried egg white was estimated to contain approximately 4 units avidin per g. 12.5 mg. were dissolved in 10 cc. of 2 per cent $(NH_4)_2SO_4$.

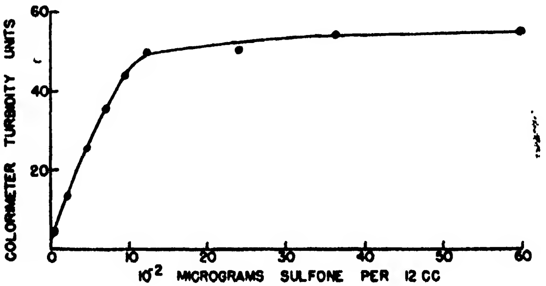

FIG. 2

A Biotin Sulfone Yeast-Growth Curve

biotin sulfone was arbitrarily compared with the activity of biotin at the level of one-fourth maximum yeast-growth increase produced by biotin. At this level of yeast growth, biotin sulfone was approximately 0.1 per cent as effective by weight as biotin. (1.5×10^{-4} γ biotin produced one-fourth maximum growth increase while 8.5×10^{-2} γ biotin sulfone produced the same amount of growth.)

Further experiments indicated that this activity was not due to traces of biotin. If the growth response had been due to contamination with biotin then the growth curve should have continued to rise in proportion to the biotin present unless, of course, the large amounts of biotin sulfone had a toxic effect on the yeast cells. The possibility of contamination with biotin was eliminated by adding 0.08 per cent biotin to biotin sulfone. With a series of concentrations of this mixture growth was obtained which did not level off at the usual "sulfone maximum" but continued to increase to the levels of growth expected from the biotin present (Table II).

TABLE II

The Effect on Yeast Growth of Adding 0.08 per cent Biotin to Biotin Sulfone

Sulfone added	Biotin added*	Yeast growth		Biotin recovered	
		Sulfone	Sulfone + biotin*		
γ	γ	*Colorimeter units*	*Colorimeter units*	γ	*per cent*
1.2	0.0010	48	132	0.00104	104
0.72	0.0006	43	101	0.00060	100
0.48	0.0004	48	83	0.00039	98
0.24	0.0002	33	70	0.00022	110
0.06	0.00005	11	46	0.00009	180**
0.012	0.00001	0	9	0.00001	100

* 0.1 γ of biotin was added to 120 γ of biotin sulfone.

** At low concentrations of biotin the addition of sulfone increased the growth. Thus, under these conditions, biotin and biotin sulfone had an additive effect on yeast growth.

The possibility that biotin sulfone is toxic was eliminated by adding 12 γ biotin sulfone to each concentration of biotin used in a standard biotin curve as shown in Fig. 3. The data of Table II show that the yeast-growth response due to biotin sulfone is not due to biotin contamination. The data in Fig. 3 further show that the relatively lower "sulfone maximum" is not due to a toxic action of larger doses of the sulfone. It is also apparent that the stimulatory effects of biotin and biotin sulfone were not strictly additive when both stimulants were added to yeast-inoculated medium, free of biotin. They exerted additive effects only when the amount of biotin was less than the concentration required to produce growth up to the level of the "sulfone maximum."

It is evident from the curve of Fig. 2 that biotin sulfone is capable of stimulating the growth of yeast cells only to a limited extent, that is, the growth increase reaches a maximum at a relatively low level. In an attempt to increase the "sulfone maximum," the concentrations of the various components of the assaying medium were increased. However, doubling the amounts of thiamine, pyridoxin, β-alanine, inositol, in-

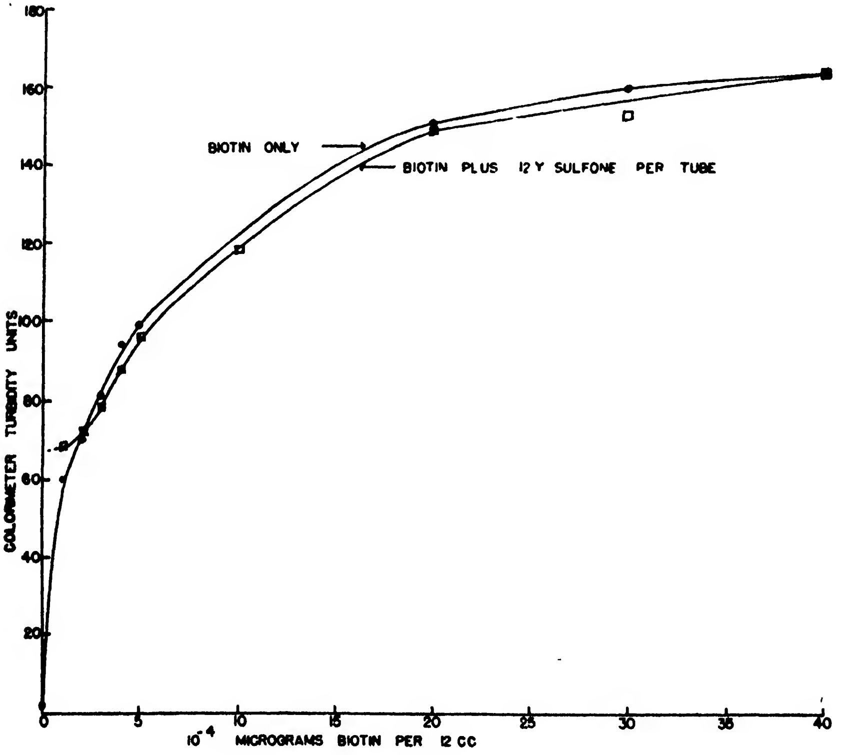

Fig. 3

Yeast-Growth Curves Illustrating that Biotin Sulfone is Not Toxic when Added to the Flasks Used for a Complete Biotin Standard Growth Curve

organic salts, or sugar did not raise the sulfone growth maximum. Likewise, the following supplements added to the basal medium were without effect: nicotinic acid, riboflavin, *d*-calcium pantothenate, choline, folic acid, asparagine, ethyl alcohol, *p*-aminobenzoic acid, pimelic acid, ascorbic acid, glucose, adenine, guanine, xanthine, uracil, a mixture of amino acids, and increased carbon dioxide tension. Nor did these

supplements when added to the medium have any demonstrable effect on the yeast growth produced by biotin. Growth was somewhat higher with biotin in the presence of the amino acid mixture than with biotin alone.

Doubling the amount of aspartic acid in the medium increased the sulfone maximum to about 65 turbidity colorimeter units but likewise increased the rate of yeast growth in the presence of biotin. As reported by Hertz (8), it was found that S.M.A. "vitamin-free" casein hydrolyzate added to the assay medium definitely increased the growth with all concentrations of biotin tested. Casein hydrolyzate added to the assay medium increased the growth with sulfone, but only in proportion to the increase of growth with biotin under the same conditions. Thus the relative growth remained the same and the relative activity was unaffected. When the assay cultures were incubated for more than 16 hours, the "sulfone maximum" increased, but this increase was again proportional to the higher maximum obtained with biotin during the same length of time.

When biotin was the growth stimulant, the yeast growth obtained when asparagine was substituted for aspartic acid was somewhat less at the lower concentrations of biotin than that obtained when aspartic acid was present in the medium. At concentrations of biotin above 3×10^{-4} γ per tube the growth with asparagine is either the same as, or greater than, that with aspartic acid. The absence of both of these amino acids definitely retarded the growth response produced at various concentrations of biotin. These yeast-growth curves are plotted in Fig. 4. It should be noticed that the growth obtained under all three conditions reaches nearly the same maximum level.

Fig. 5 shows the effect of these amino acids on the growth-promoting activity of biotin sulfone. When neither aspartic acid nor asparagine was present there was no growth. There was almost no growth with asparagine in the presence of sulfone.

In the Cleveland laboratory, asparagine (1.0 mg. in 10 cc. of basal medium) gave no growth while double that amount produced some growth, whereas in the Cornell laboratory a slight growth response, varying between 10 and 15 colorimeter units was always obtained.[2] *dl*-Aspartic acid was less satisfactory than *l*-aspartic acid for yeast

[2] In the Cornell laboratory an alteration was encountered in the yeast which grew almost as well with sulfone in the presence of asparagine as in the presence of aspartic acid.

growth, but doubling the amounts of *dl*-aspartic acid produced the same effect as the usual amount of *l*-aspartic acid. Glutamic acid did not sub-

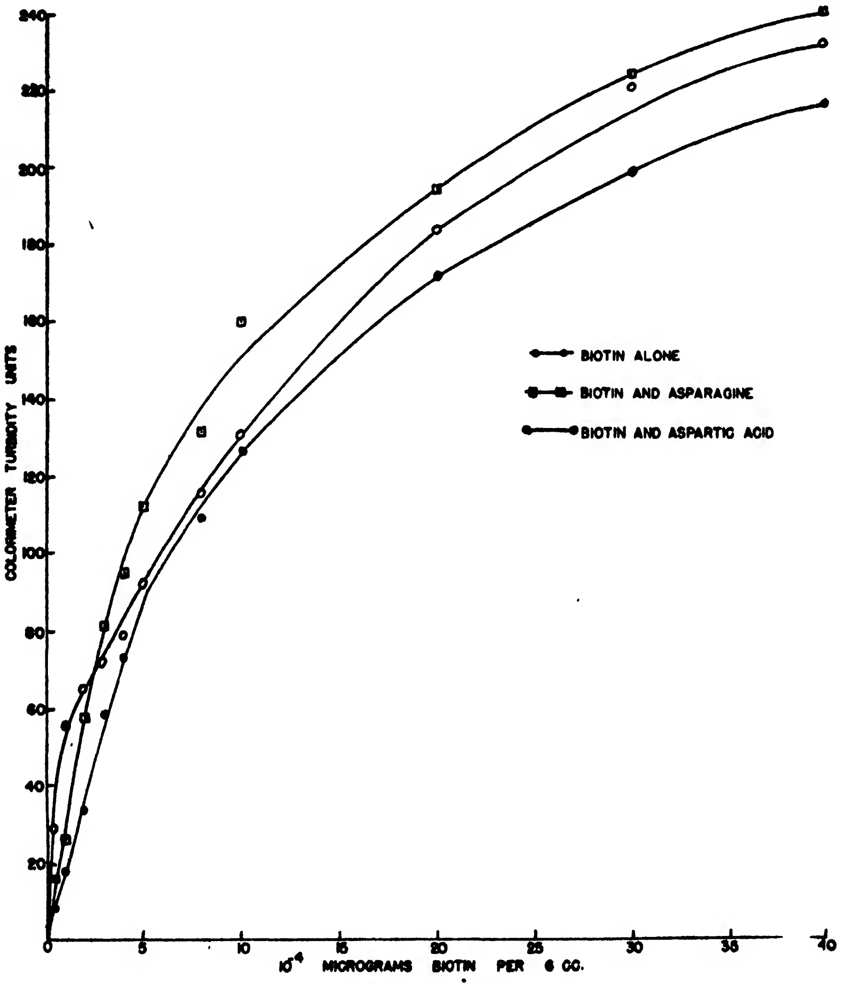

FIG. 4

Yeast-Growth Curves Illustrating the Effects of Aspartic Acid and Asparagine on the Yeast-Growth-Promoting Activity of Biotin

stitute for aspartic acid to stimulate growth in the presence of biotin sulfone. Thus, the growth-promoting action of aspartic acid with biotin sulfone seems to be quite specific.

Biotin sulfone was stable to heat sterilization. When flasks containing the amounts of sulfone required for a typical sulfone growth curve in 2 cc. were sterilized for 20 minutes in a steam sterilizer, there was no apparent decrease in activity.

Avidin and Biotin Sulfone. Sufficient avidin completely inhibited the activity of biotin sulfone. Since 1 unit of avidin inhibits 1 γ of biotin (7), 0.88 units of avidin should inhibit 1 γ of biotin sulfone if the inhibition due to avidin is based on a combination in molecular ratio. Table III shows the amounts of avidin required for each concentration of sulfone for a typical growth curve. Since avidin was added in a solution of 2 per cent ammonium sulfate, 0.5 cc. of 2 per cent ammonium sulfate was added to a series of control flasks and found to have no effect on the

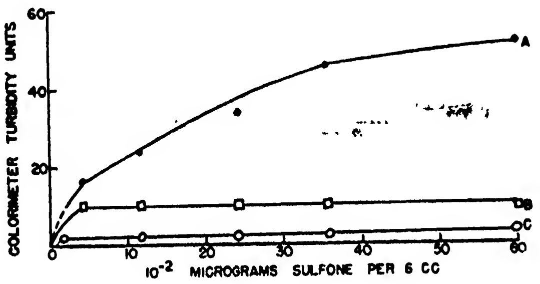

FIG. 5

Yeast-Growth Curves Illustrating the Effects of Aspartic Acid (Curve A), Asparagine (Curve B), and Glutamic Acid (Curve C) on the Yeast-Growth-Promoting Activity of Biotin Sulfone

growth (Table III). When avidin was added to a mixture of sulfone and biotin it exhibited an equal affinity for both compounds.

A limited amount of avidin added to excess biotin sulfone, instead of partially inhibiting the yeast growth, resulted in greatly increased yeast growth. In fact, the growth maximum was characteristic of biotin stimulation which, as stated above, is much higher than any growth maximum ever attained with the sulfone as the stimulant. The results of one such experiment are tabulated in Table IV. Examination of the data of Table IV reveals that avidin in excess of the amount required by the sulfone present completely inhibited the yeast growth, but as soon as the sulfone was in excess, the yeast growth far exceeded the maximum growth expected from the sulfone in the absence of avidin. The avidin concentrate, prepared from frozen egg white by a method similar to that described by Eakin, Snell, and Williams (7) contained free avidin and

some avidin-biotin complex (AB). When excess sulfone was added to a given amount of avidin concentrate, the sulfone displaced the biotin

TABLE III

The Effect of Excess Avidin on the Yeast-Growth-Stimulating Effect of Biotin Sulfone

Sulfone added	Yeast growth		
	No avidin	0.63 unit avidin*	No avidin + 0.5 cc. of 2% $(NH_4)_2SO_4$
γ	*Colorimeter units*	*Colorimeter units*	*Colorimeter units*
0.60	53	3	
0.36	53	3	55
0.24	50	0	50
0.12	49	0	50
0.096	43	2	50
0.072	35	0	
0.048	26	1	
0.024	13	0	

* 6.28 mg. of an avidin concentrate containing 2000 units per gram were dissolved in 10 cc. of 2 per cent ammonium sulfate and 0.5 cc. of this solution added to each flask before the volume was made up to 2 cc.

TABLE IV

*The Effect of Excess Sulfone on a Limited Amount of Avidin Concentrate**

Sulfone added	Avidin required	Yeast growth	
		Without avidin	With avidin**
γ	*Units*	*Colorimeter units*	*Colorimeter units*
12.0	10.6	50	145
6.0	5.3	50	143
1.2	1.06	55	148
0.6	0.53	49	3
0.36	0.32	45	3
0.24	0.22	44	0
0.12	0.11		0
0.072	0.06		3
0.024	0.02		3

* An avidin concentrate containing 540 units per gram was added in 2 per cent $(NH_4)_2SO_4$.

** The avidin concentrate added to each flask contained 0.54 unit of avidin.

of the AB complex, and the biotin thus displaced accounted for the stimulation which produced the maximum yeast-growth increase.

Since biotin sulfone did not stimulate the growth of yeast in the absence of aspartic acid, the yeast growth obtained with a medium containing neither aspartic acid nor asparagine cannot be due to biotin sulfone and must therefore be due to biotin. Control experiments with mixtures of biotin and biotin sulfone demonstrated that the biotin content could be quantitatively determined with this modified medium since a correction was not needed for the amounts of biotin sulfone present.

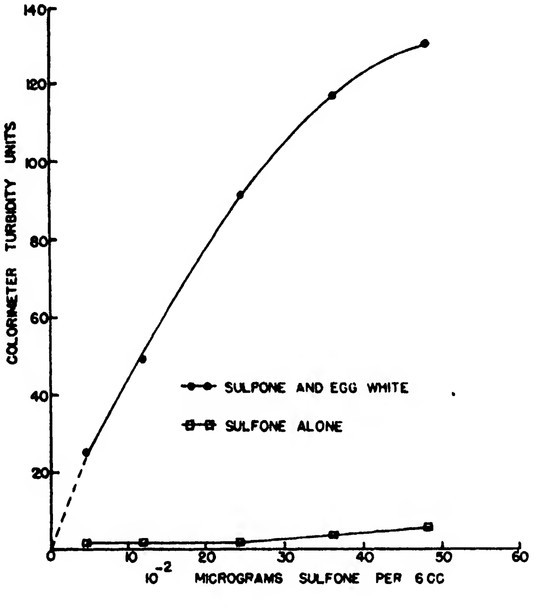

FIG. 6

Yeast-Growth Curves Illustrating the Effects of Fresh Egg White (0.125 cc. per Tube) on the Growth-Stimulating Properties of Biotin Sulfone in a Medium Free of Aspartic Acid

Since the presence of biotin sulfone did not affect the determination of biotin when a medium free of aspartic acid and asparagine was employed, we were able to measure quantitatively the liberation of biotin from the avidin-biotin complex of avidin concentrates, dried egg white preparations, and fresh egg white. Fig. 6 shows the curves obtained with sulfone in a medium free of aspartic acid with and without the addition of fresh egg white.

The amount of biotin liberated from the AB complex by sulfone de-

TABLE V

The Effect of Biotin Sulfone on the Bound Biotin of Various Avidin Preparations

Sulfone added	Free avidin added		AB-biotin added **	Biotin liberated† from AB-biotin	
γ	*Units*	*Sample No.**	γ	γ	*per cent*
4.0	0.0362	24	0.0040	0.00110	27.5
0.4	0.0362	24	0.0040	0.00059	14.7
0.2	0.0362	24	0.0040	0.00050	12.5
4.0	0.0362	24	0.0370‡	0.00186	5.0
1.2	0.1100	24	0.0120	0.00156	13.0
1.2	0.0110	24	0.0012	0.00068	56.7
1.2	0.0110	24	0.0112‡	0.00087	7.8
1.2	0.0250	219-2	0.0040	0.00045	11.2
1.2	0.0250	267-1	0.0021	0.00027	12.8
1.2	0.0070	21	0.0009	0.00004	4.4
1.2	0.112	A	0.0125	0.00230	18.4
0.48	0.112	A	0.0125	0.00142	11.4
0.48	0.045	A	0.0050	0.00064	12.8
0.24	0.112	A	0.0125	0.00072	5.8
0.24	0.045	A	0.0050	0.00051	10.2
0.048	0.045	A	0.0050	0.00021	4.2
1.2	0.0064	B	0.0012	0.00038	31.6
0.12	0.0064	B	0.0012	0.00032	26.6
1.2	0.0107	B	0.0020	0.00078	39.0
0.12	0.0107	B	0.0020	0.00049	24.5
1.2	0.0060	C	0.0017	0.00046	27.0
0.12	0.0060	C	0.0017	0.00036	21.2
1.2	0.0100	C	0.0028	0.00076	27.2
0.12	0.0100	C	0.0028	0.00058	20.7

* Avidin preparations used in this study had the following activities:

Sample No.	Preparation	Free avidin *units/mg.*	AB-biotin *γ/mg.*
24	Avidin concentrate	0.55	0.06
219-2	" "	1.00	0.16
267-1	" "	1.00	0.085
21	" "	0.07	0.009
A	Fresh egg white	0.00090	0.00010
B	Dried egg white (B. and A.)	0.0043	0.008
C	" " " (Merck)	0.0040	0.0011

** The amount of bound biotin present in the avidin added.

† Measured as yeast growth on a medium free of aspartic acid.

‡ Extra biotin was added to the avidin solutions to neutralize most of the avidin present.

pended upon the total amounts of biotin sulfone, avidin, and biotin present, upon the excess of biotin sulfone over avidin, and the degree of saturation of the avidin with biotin. In general, the fraction of the total bound biotin replaced by sulfone was directly proportional to the ratio of sulfone to AB biotin. The data of Table V illustrate these points.

Activity of Biotin Sulfone in Treatment of Egg White Injury in Rats. Biotin sulfone shows about the same activity in curing egg white injury in rats as in promoting yeast growth.[3] Rats were fed a raw egg white diet similar to that used in previous work of György (9) except that a mixture of pure vitamins was substituted for the yeast extract. With this diet the minimum curative dose of biotin is 0.1 γ injected daily. On the basis of the yeast growth experiments in which biotin sulfone showed 0.1 per cent of the activity of biotin, 100 γ of it were injected daily for 28 days in animals with advanced symptoms of egg white injury. Healing was slow to start and there was almost no improvement for two weeks. After that period, however, healing progressed rapidly, and the animals had almost normal coats in four weeks and continued to improve after treatment was discontinued.

Discussion

Biotin sulfone stimulates yeast growth but not to as great an extent as biotin. With the diaminocarboxylic acid derivative of biotin (1), maximum growth can be obtained by increasing the concentration of the derivative to 10 times the level of biotin required. The results of the experiments described in this paper definitely show that the decreased activity of biotin sulfone cannot be compensated for by larger doses. The observation that biotin sulfone does not stimulate yeast growth in the absence of aspartic acid, whereas biotin does, is an indication that the activity of biotin sulfone is not due to a limited synthesis of biotin from the sulfone. The sulfone activity seems to be limited to only a portion of the yeast-growth-promoting functions of biotin.

The inhibition of biotin sulfone activity by avidin indicates that the oxidized form of the sulfur in the biotin molecule does not interfere with the interaction between biotin and avidin. The displacement of biotin from the avidin-biotin (AB) complex by excess sulfone is further evidence

[3] This is in contrast with the behavior of diaminocarboxylic acid which, although about 10 per cent as active as biotin in promoting yeast growth, is completely ineffective in treatment of egg white injury in doses up to 200 times the minimum curative biotin dose.

that the biotin sulfone still retains the essential functional group or groups for the reaction between biotin and avidin.

Summary

Biotin sulfone, derived from biotin by oxidizing the sulfur to the corresponding sulfone, stimulated the growth of yeast but even with large amounts of sulfone the yeast growth did not increase above a relatively low maximum which was only about one-third of the maximum growth obtained with biotin. Biotin sulfone was found to be approximately 0.1 per cent as effective as biotin when the activities were compared at the level of one-fourth maximum growth increase due to biotin.

Biotin sulfone did not stimulate the growth of yeast when aspartic acid was absent from the medium; asparagine could not be substituted for aspartic acid.

Avidin combined with sulfone when avidin was present in excess.

Excess biotin sulfone liberated biotin from the avidin-biotin complex of avidin preparations, dried egg white, and fresh egg white.

An avidin assaying method for measuring the biotin-combining activity of avidin-containing preparations has been described.

The authors wish to express their appreciation to Miss Carol Tompkins of the Cornell laboratory for assisting in the technical work.

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Nutritional Value of Bread Containing Soya Flour and Milk Solids[1]

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Introduction

A major improvement in the quality of the American dietary has been brought about by the enrichment of white bread with the minerals and vitamins commonly deficient in the dietary and for which bread is a proper carrier. In this enrichment program little or no effort has been made to improve the quality or quantity of the bread protein, nor have protein standards been proposed in the definition of enriched bread (1) by the United States Food and Drug Administration.

Other countries, especially Canada and Great Britain, have preferred to improve bread by the use, sometimes compulsory, of cruder flours from which less of the various nutrients have been removed. Chick (2) has shown that the protein of the "National Wheat Meal" flours is approximately 15% better than that of white flour, while the protein of whole wheat meal is some 20% better. It is evident that an effort should be made to improve the protein of white bread at least until it is nutritionally equivalent to whole wheat bread.

For some time skim milk solids have been used in white bread formulae to improve the taste and appearance. This use of milk solids has been attractive also because the calcium, riboflavin, and protein content of white bread is significantly improved when more than 3% is used in the bread formula. Jones and Devine (3) have reported that soya, peanut, and cottonseed flours markedly improve the protein of white flour, and attribute this improvement to the lysine in these added flours.

This year protein supplies are limited, and bread must become increasingly important in the American dietary. Because of the shortage of

[1] Aided by a grant from the General Baking Company.

milk solids, bakers are forbidden to use in their bread formulae the 6% milk solids necessary to bring the protein up to whole wheat levels (4). Soya flour has been suggested as an alternate for milk solids in bread. It has been used as a constituent of bread for many years.

The present investigation was undertaken because there was little information on the value of soya flour in combination with, or even in replacement of, milk solids in bread. Since the diets fed to each group of animals were nutritionally complete, and since the nitrogen content of each diet was the same (1.8%), the differences in growth observed in these experiments may be used to compare the quality of the proteins.

EXPERIMENTAL

A commercial bakery collaborated in the preparation of the eight separate samples of bread used in these experiments. The quantity of white flour, water, yeast, arkady,[2] salt, lard, sugar, and malt was kept constant. The skim milk solids

TABLE I

*Analyses of Bread Crumbs**

Sample	% Moisture	% N dry wt.	% N wet wt.	g. per 1.8 g.N
I	13.02	2.51	2.18	82.5
II	12.08	2.61	2.29	78.5
III	12.98	2.62	2.28	78.8
IV	12.28	2.67	2.34	76.9
V	13.75	2.66	2.30	78.2
VI	12.70	2.69	2.35	76.5
VII	12.31	2.73	2.40	75.0
VIII	12.01	2.72	2.39	75.3

content varied between 0% to 6%, and the full-fat soya flour content varied from 0% to 5% of the weight of the flour in the bread formula. All breads were enriched with vitamins and minerals in accordance with Food Distribution Order Number One (5) at levels corresponding to existing flour standards (6). Although each batch contained 300 pounds of flour, only the central 75 loaves of bread were taken from each run for use in this research.

* It is customary to calculate the protein content by multiplying the nitrogen value by 5.7 for wheat flour, by 6.38 for milk solids, and by 6.25 for soya flour. A diet containing 10% protein would contain approximately 1.8% $\left(\frac{10 \times 100}{5.7}\right)$ nitrogen. All diets were formulated on an equi-nitrogen basis.

[2] Arkady is a proprietary compound used as a yeast food and contains calcium sulfate, ammonium chloride, potassium bromide, sodium chloride, and wheat flour.

TABLE II

Diet Components

Diet		I	II	III	IV	V	VI	VII	VIII
Hydrogenated fat.		10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Salt mixture*		4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Corn starch		3.5	7.5	7.2	9.1	7.8	9.5	11.0	10.7
Bread crumbs	I	82.5							
	II		78.5						
	III			78.8					
	IV				76.9				
	V					78.2			
	VI						76.5		
	VII							75.0	
	VIII								75.3
Totals		100	100	100	100	100	100	100	100

Supplement A: 4 drops every 4 days to each rat of each group
Supplement B: 4 drops every 4 days to each rat of each group

Supplement A

Each drop of alcohol-aqueous solution to contain:

Thiamin	0.020 mg.
Riboflavin	0.025 "
Ca pantothenate	0.100 "
Pyridoxin	0.020 "
Niacin	0.100 "

Supplement B

One drop of olive oil to contain:

Vitamin A	60 I.U.
Vitamin D	6 I.U.
α-Tocopherol	0.01 mg.

* Hubbell, *J. Nutrition* **14**, 273 (1937).

TABLE III

Weight Increase in Terms of Food Consumption

Group	Bread formula: Milk solids	Bread formula: Soya flour	Averages during 56 day period: Gain in weight	Averages during 56 day period: Food eaten	Gain per 100 g. food	Gain per g. nitrogen	
	%	%	(g.)	(g.)	(g.)	(g.)	ratio
I	0	0	18.7	327.1	5.72	3.17	1.00
II	4	0	27.9	371.1	7.52	4.17	1.32
III	0	3	26.6	339.9	7.82	4.33	1.37
IV	1.5	2.5	29.3	353.2	8.30	4.61	1.45
VI	0	5	28.1	332.5	8.45	4.69	1.48
VII	6	0	29.9	351.8	8.50	4.72	1.49
VIII	2.3	3	32.5	349.1	9.31	5.17	1.63
V	3	3	33.7	360.3	9.35	5.20	1.64

The loaves were sliced and spread two days to dry in a darkened room, then crumbed, and analyzed for nitrogen and moisture content (7). On the basis of the analytical results in Table I, eight diets were prepared as shown in Table II for feeding to laboratory animals.

A total of 160 month-old albino male rats of the Wistar strain were divided into 8 groups of 20 rats each, so that litter mates were well distributed. The average weight of all groups was 60 to 61 grams at the start of the experiment. Each rat was housed separately in a galvanized wire cage with a raised floor and supplied *ad libitum* with diet and tap water. During the 59 day test period each rat was weighed twice weekly, and records were kept of individual food consumption.

The influence of these diets on the growth and development of the rats is shown in Table III. Since food consumption has an influence upon weight increase, the data have been expressed in terms of food and food nitrogen intake.

Discussion

During the first several days the animals in all groups showed a temporary loss in weight, presumably an adjustment to the new type of diet. Subsequently all rats showed a progressive weight increase until the end of the experiment. While it is satisfactory to compare the beginning with the final weight in calculating the weight increase in the fourth column of Table III, it seemed more correct to compare the lowest weight (at the end of three days) with the final weight.

Of the eight breads which were fed, the bread containing 3% soya flour and 3% skim milk solids (V) produced the best growth effects. The protein in this bread was thus superior to that of a bread containing 6% skim milk solids (VII) or 5% soya flour (VI).

The protein contributed by 6% skim milk solids is approximately the same as that contributed by 5% soya flour. There was no significant difference between the growth effects of these two breads containing these amounts of skim milk solids (VII) and soya flour (VI). The superior values of blended proteins were shown also in the case of the bread containing 1.5% skim milk solids and 2.5% soya flour (IV) as compared to one containing 4% skim milk solids (II) or 3% soya flour (III). As was to be expected, the bread containing neither soya flour nor skim milk solids showed the poorest growth of all.

A bread containing 2.3% skim milk solids and 3% soya flour (VIII) was superior in nutritional value to a bread containing 6% skim milk solids (VII). Since the total protein in these breads was approximately the same, it appears that a bread containing equal quantities of soya flour and skim milk solids is superior in protein quality to one containing the same amount of protein added as skim milk solids only.

Summary

1. The superior nutritive value of bread containing soya flour has been demonstrated. Soya flour supplements the proteins of milk in the bread formula.

2. A bread containing 3% skim milk solids and 2.3% full fat soya flour was superior to a bread containing 6% milk solids not fat.

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Electrophoretic Studies with Plant Viruses

I. Tobacco Mosaic Virus

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Introduction

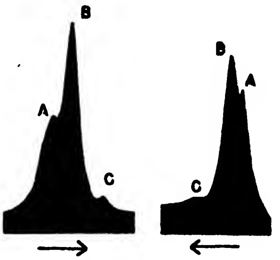
The indications from the Longsworth scanning patterns (1) obtained on the electrophoresis of extracts from healthy tobacco plants (*Nicotiana tabacum*) and of extracts from tobacco plants infected with tobacco mosaic virus are that the normal proteins extracted by the methods used in this investigation are not materially affected by the development of the disease. There is an additional component which appears in the extracts from the diseased plants, as evidenced by the patterns, and its appearance is correlated in time with the appearance of the symptoms in the infected plants. It is believed, for reasons that will be given subsequently, that the material in the extracts from the diseased plants which gives rise to this particular abnormality in the scanning patterns is the virus.

The difficulty in the electrophoresis of fresh leaf extracts has been in the preparation of the sample. Invariably there is much pigment in the expressed sap, and generally the concentration of the various compounds is too low for satisfactory scanning. The following method has been found useful in the preparation of fresh leaf material for electrophoresis, and was the method used in this study.

Methods

The harvested leaves were cytolyzed with ether (2) and the vacuolar sap was pressed out with a hydraulic press and discarded. The press-cake was then suspended in water and the excess water subsequently removed with the press. The pressed material was washed three times in this manner before it was ground in a food chopper, after which it was transferred to a Waring blender and reground in

the presence of 0.10 M phosphate buffer at pH 7.5. The vacuolar sap contains little or no protein and no virus, but it does carry much pigment and soluble non-protein-material. The successive washings with water do not affect the character of the patterns, but they do effect the removal of additional pigment. Concentration of the proteins was effected by repeated use of the same buffer in the extraction of successive portions of the leaf material; thus approximately 100 ml. of buffer was used in the extraction of 600 g. of fresh leaves. Further concentration was obtained by placing the buffer extract in a celophane bag, and hanging it before a fan.



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30 minutes electrophoresis

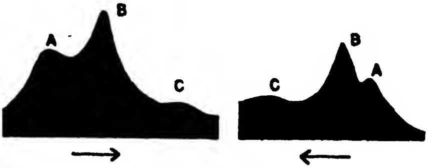

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Fig. 1

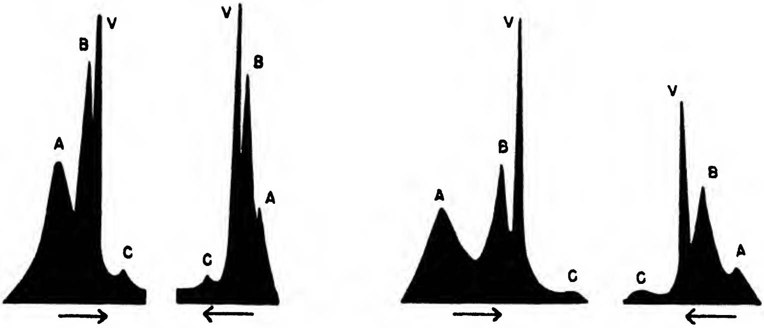
Electrophoretic Patterns of Extracts Obtained from Healthy Tobacco Leaves
0.10 M phosphate buffer at pH 7.5 was used

The larger leaf fragments were removed by straining the extract through cheesecloth, smaller fragments and some starch by centrifugation at low speed, and finally the plastids and other bodies were removed by centrifugation at 16,000–25,000 r.p.m. for 2–3 minutes. In some instances high speed centrifugation was carried out after dialysis against the buffer (pH 7.5) which was to be used in the electrode compartments of the apparatus. Dialysis was carried on for about 18 hours at 4°C.

Illumination was obtained with an incandescent lamp. Infra-red sensitive film was used, and a Wratten F filter was interposed between the camera and the slit. A potential gradient of 1.7 volts per centimeter was used.

Results

The patterns in Fig. 1 were obtained with extracts from healthy tobacco leaves after 30 and 60 minutes of electrophoresis. No differences have been observed in the patterns from very young leaves and from



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30 min. electrophoresis

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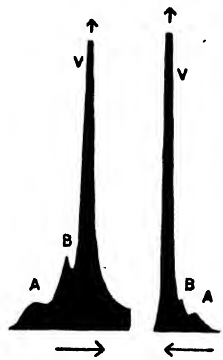
60 minutes electrophoresis

Fig. 2
Electrophoretic Patterns of Extracts Obtained from Tobacco Leaves After the Plants Had Been Infected with the Tobacco Mosaic Virus 9 days
0.10 M phosphate buffer at pH 7.5 was used



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30 minutes electrophoresis

Fig. 3
Electrophoretic Patterns of Extracts Obtained from Tobacco Leaves After the Plants Had Been Infected with Tobacco Mosaic Virus for 15 days
0.10 M phosphate buffer at pH 7.5 was used

leaves over three months old. Apparently the composition of the extracts is a constant characteristic of the healthy tobacco plant.

The appearance of an abnormality in the scanning patterns of extracts from diseased plants is correlated with the occurrence of symptoms in

the plants, since the patterns obtained with the extract from leaves harvested five days after inoculation, at a time when no symptoms were evident, was identical with that obtained from the normal plants. Vein clearing was evident on the ninth day after inoculation. The patterns obtained with the leaves harvested at this time are shown in Fig. 2 (time for electrophoresis was 30 and 60 minutes). Components A, B, and C in Fig. 2 were identified from their migration velocities as being identical with the corresponding components in Fig. 1. Component V is new.

Symptoms of the disease had fully developed after fifteen days. The patterns obtained with these leaves which showed fully developed symptoms is presented in Fig. 3. The normal components A and B were still present in the extracts, which in this case were more dilute than for Figs. 1 and 2.

The question may be raised, particularly with reference to the older leaves, relative to the extent of the invasion of the plant by the virus after fifteen days, and to the possibility that a mixture of diseased and healthy tissue may have been involved in the determinations with these particular extracts. By way of an attempt to get an answer to this question these plants were cut back, and two shoots from each were permitted to grow so that the opportunity for systemic invasion by the virus might be optimum. The shoots were harvested two weeks later, and the scanning patterns were determined. The patterns obtained with these shoots were essentially identical with the one given in Fig. 3, although component C appeared in some of the preparations which were concentrated to a greater degree.

Discussion

The results obtained in this investigation are about what one would have anticipated, although there may be some surprise at the simplicity of the patterns from the healthy plants, and one might have expected a change in the patterns with the age of the plant.

With respect to the quantitative determination of the concentrations of components A, B, and C in both the infected and healthy plants, the mode of extraction precludes precise measurements. The feeling has developed, however, as a result of dealing with many samples, that there is no substantial change in the quantities of these components per unit weight of plant with the development of the disease in the tobacco plant.

The evidence from these data that the development of tobacco mosaic virus in the tobacco plant is not associated with any material change in the composition of the proteins normally present is in agreement with

the evidence from serological studies, where antibodies engendered in the blood stream of experimental animals by sap from diseased plants are precipitated by the sap from healthy plants (3).

There can be little doubt that the component V is the virus. The reasons for this belief are threefold, namely, component V occurs only in the diseased plant and increases in quantity as the disease progresses, the area blocked out in the pattern by this particular component in the instance of the plants infected for the longer periods is more than thirteen times larger than the areas blocked out by the other components (4), and the narrowness and the height of the blocked out area indicates that the component V has a very small diffusion constant, which is characteristic of the tobacco mosaic virus (5).

These data, and data which have been obtained with other sap-transmissible viruses, and which are in general agreement with these for tobacco mosaic virus (with the exception of the degree of dominance of the virus component) give rise to the expectation that electrophoresis may serve as a valuable adjunct in diagnosis involving virus diseases in plants. This will be particularly true if the behavior of the various viruses in the electrophoresis cell is independent of the host.

Summary

1. The Longsworth scanning patterns obtained with the extracts from healthy tobacco leaves at pH 7.5 indicate that there are three protein components in the extracts.

2. The composition of the extracts from the healthy leaves is not affected by the age of the plants up to three months.

3. An additional component appears in the extracts of tobacco plants infected with tobacco mosaic virus, and its appearance is correlated in time with the appearance of symptoms in the infected plants. The additional component is believed to be the virus.

4. The development of the disease does not appear to effect any change in the nature or concentration of the normal proteins.

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Studies on the Oxidation of the Aromatic Amino Acids Tyrosine, Tryptophan, and Phenylalanine[1]

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Introduction

Although there is a large literature dealing with the metabolism of the aromatic amino acids and with the key rôle that some of them play in enzyme reactions, the literature on the chemical oxidation of these acids is rather scant. It has been stated that tyrosine and tryptophan are both probably responsible for some of the reducing action of proteins (1), but the reactions involved have not been demonstrated except that treatment of proteins with iodine followed by hydrolysis has been shown to produce iodo- and diiodotyrosine (2, 3). The work of Bowman (4) indicates that the rate at which tyrosine is oxidized by some reagents is greatly dependent upon the salts present in solution, but the mechanism of the salt effect has not been explained. Recently, however, Johnson and Tewkesbury (5) have studied the hypoiodous acid oxidation of diiodotyrosine to thyroxine and have suggested a mechanism to explain the reaction. The present work was undertaken to study potentiometrically the reactions of tyrosine, tryptophan, and phenylalanine with various oxidizing agents and, if possible, to isolate products from these reaction mixtures. The work is not completed, but since it is not being continued at present some of the results are presented.

Experimental

The amino acids, potassium permanganate, and cerox (ceric ammonium sulfate) were C. P. grade commercial products. The potentials were measured at blank platinum electrodes employing for reference, calomel half-cells containing 3.5 *M* potassium chloride solution. All measurements were made in a well stirred air bath at 30.0° ± 0.1°C. The potentials were recorded by a Leeds and Northrup type K_2 potentiometer employing a type R (No. 2500-b) galvanometer as a null instrument.

The first tests were run to determine whether tyrosine, tryptophan, and phenylalanine were oxidized by such oxidants as potassium permanganate and cerox. It was found that in a 0.5 *M* phosphate buffer of

[1] The authors are indebted to the Buhl Foundation for a research grant in support of this investigation.

Contribution No. 518 from the Department of Chemistry, University of Pittsburgh.

pH 6.4 tyrosine and tryptophan were readily oxidized by permanganate. A mixture of either amino acid and oxidant imparted a charge to a platinum electrode, but the potential drifted rapidly with time. In an attempt to stabilize the potentials solutions were run at various pH values. The most stable potentials were obtained in strongly acid solutions. Such acid solutions are the only ones considered in this report.

Fig. 1 shows the potentials observed when phenylalanine, tyrosine, and tryptophan were titrated with potassium permanganate in 0.5 *N* sulfuric acid. It was apparent that phenylalanine was not oxidized and could serve as a control while tyrosine and tryptophan gave much lower potential values. The lowered potential values are evidence that the oxidant was reacting with the amino acids. Such a conclusion would have to be reached by the simpler procedure of merely observing the color of the potassium permanganate, but the potential measurements have certain advantages as will appear below. The two curves shown for tyrosine, and the two for tryptophan give a measure of the drift of the potentials with time. It is apparent that no end point was reached during the addition of four equivalents of oxidant.

Fig. 2 shows the potentials obtained on titrating tyrosine, tryptophan, and phenylalanine with cerox in 0.5 *N* sulfuric acid. It was apparent in this case also that phenylalanine was not oxidized while tyrosine and tryptophan were. The curve for tyrosine shows a marked break at three equivalents and is quite different from that obtained with permanganate. The two curves for each amino acid show the drift of the potential with time.

In order to check the disappearance of tyrosine the Millon test was run on tyrosine solutions treated with different amounts of cerox. The results are shown in Fig. 3. The test suffers from the fact that it is not strictly specific for tyrosine so that the oxidation products of tyrosine may respond to some extent. At any rate, the test shows that as the cerate was increased, the Millon reaction decreased and became practically negative when three equivalents of oxidant had been added. The agreement between the disappearance of the Millon reaction and the formation of a potentiometric end point is striking. In carrying out the Millon test it was observed that the addition of mercuric sulfate to the solutions containing more than one equivalent of cerate resulted in the formation of a murky solution. A tan flocculent precipitate settled on heating the murky solution. In the usual determination of tyrosine in protein hydrolyzates tryptophan is removed at this point in the procedure as a mercury complex similar in appearance to the observed precipitate. The formation of such a precipitate suggests that an indole compound may have been formed in the oxidation of tyrosine.

It seemed of interest to isolate this mercury precipitate and learn something of its properties. From 1.81 g. of tyrosine 1.88 g. of mercury precipitate were obtained. After drying at reduced pressure over sulfuric

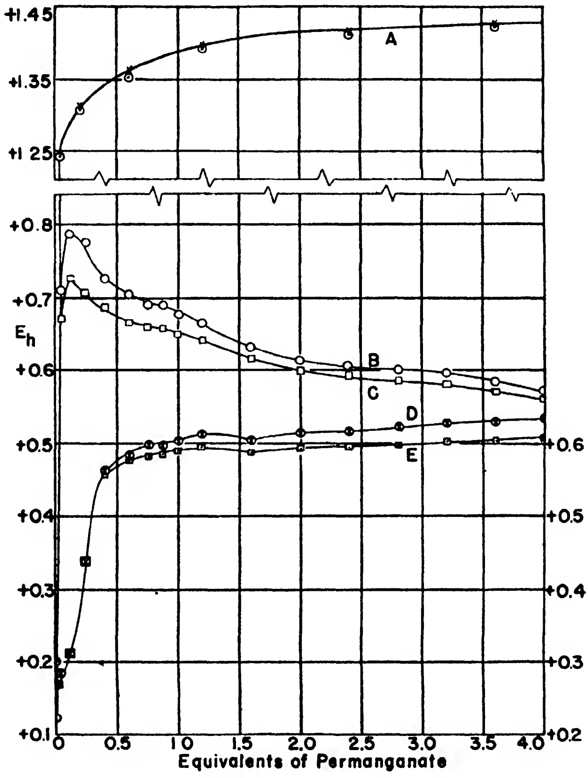


Fig. 1

Potentiometric Titrations of Phenylalanine, Tyrosine, and Tryptophan with Potassium Permanganate

The points × and ⊙ on Curve A show the potentials of the phenylalanine system 10 and 15 minutes after addition of the oxidant. Curves B and C are for tyrosine, and D and E for tryptophan. The potentials of B and D were measured 10 minutes, and C and E 15 minutes following oxidant addition. The ordinate scale for phenylalanine and tyrosine is at the left, for tryptophan at the right. The titrations were run on 50 ml. portions of 0.001 *M* solutions of the amino acids in 0.5 *N* sulfuric acid using 0.04 *N* permanganate as the oxidant.

acid it contained 23.8 ± 0.7 per cent mercury and 3.55 ± 0.01 per cent nitrogen. These figures suggest that the material contained a ratio of two nitrogen atoms to one atom of mercury, but both figures are too low

to be explained by a mercury salt of tyrosine, or some simple oxidation product of it. The fact that only 47.6 per cent of the added nitrogen was recovered suggests that only half of the nitrogen was converted into

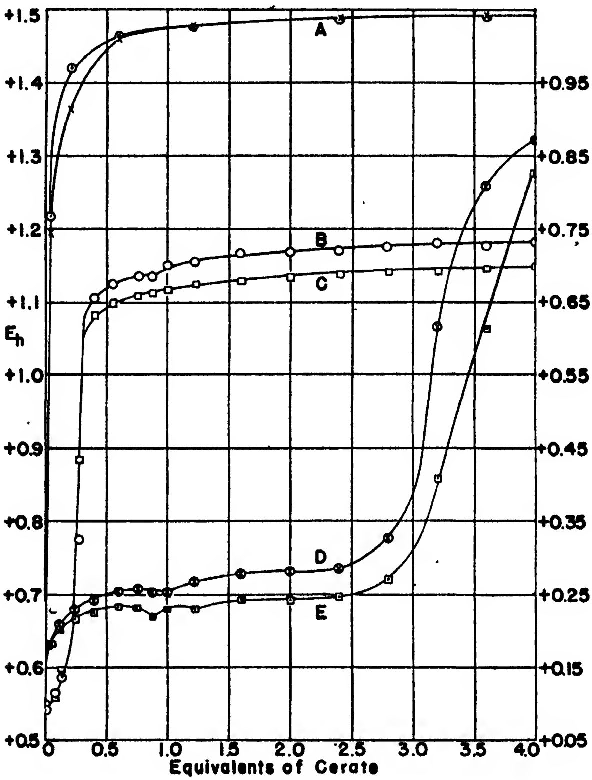


FIG. 2

Potentiometric Titrations of Phenylalanine, Tyrosine, and Tryptophan with Cerox

The points × and ⊙ on Curve A show the potentials of phenylalanine 5 and 10 minutes after addition of the oxidant. Curves B and C are for tryptophan, and D and E for tyrosine. The potentials of B and D were measured 5 minutes, and C and E 15 minutes following oxidant addition. The ordinate scale for phenylalanine and tyrosine is at the left, for tryptophan at the right. The conditions are the same as in Fig. 1 except that 0.04 *N* cerox was the oxidant.

this particular compound. After removal of the mercury by hydrogen sulfide the material remaining was precipitated with alcohol and dried. It was insoluble in alcohol, acetone, and ethyl acetate and only slightly

soluble in water. It was much more soluble in acids and bases. Attempts to crystallize the material were not successful. Three different preparations showed 4.76, 5.52, and 4.92 per cent nitrogen. The change in nitrogen content due to the hydrogen sulfide treatment corresponds roughly to the removal of mercury and suggests that the rest of the material contained in the mercury salt remained intact. Despite the variation in nitrogen content the three preparations showed the same amount of color with the Folin phenol reagent (29.5 per cent of the color given by an equal weight of tyrosine). The ninhydrin reaction was very faint in each case so apparently no free α-amino acid group was present. Combustion of the compound left only a trace of ash. The absorption spectra, run on a Beckman spectrophotometer, are shown in Fig. 4. It is apparent that the three preparations were very similar and that they were quite

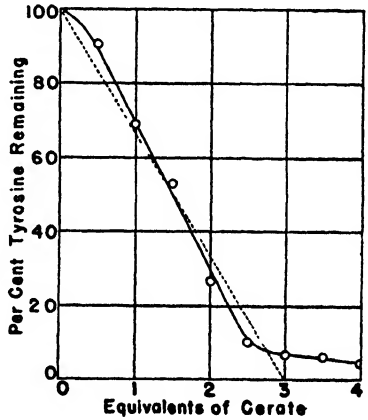


FIG. 3

Disappearance of the Millon Phenol Reaction on Oxidizing Tyrosine with Cerox

The solid curve represents the decrease in color of the Millon reaction observed with the Evelyn photoelectric colorimeter when 0.001 M tyrosine in 0.5 N sulfuric acid was oxidized with 0.04 N cerox. The color is plotted as per cent tyrosine remaining [100 × (2 − log G)/(2 − log G_1) where G and G_1 are the galvanometer readings for the oxidized and unoxidized solutions]. The broken line indicates the theoretical curve for a linear, three equivalent reaction.

different from tyrosine. The curves resemble that for indole-3-carboxylic acid (6) more closely than they resemble any other absorption curve found in the literature. No curve could be found for indole-2-carboxylic acid.

The effect of adding some mediators to the tyrosine and tryptophan solutions before oxidation was tested with the following results. Ferric sulfate (5×10^{-5} M) had no marked effect, but did cause a slight stabilization of the potentials. Addition of benzoquinone, purified by steam distillation and recrystallization from petroleum ether, gave a quite different effect when added to the tyrosine system in a concentration of 5×10^{-5} M. The level of the potential was not changed, but the apparent end point was shifted to two equivalents. No measurable effect other than some stabilization of the drifting potentials was noted on adding

benzoquinone to the tryptophan system. The addition of ascorbic acid as a mediator gave results indicative of some interaction of it or its oxidation products with both tyrosine and tryptophan. The reactions involved were not studied further.

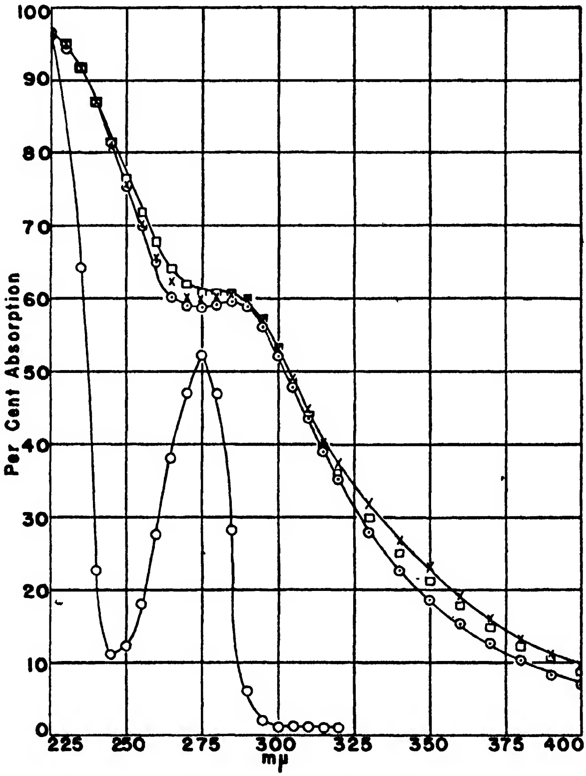


FIG. 4

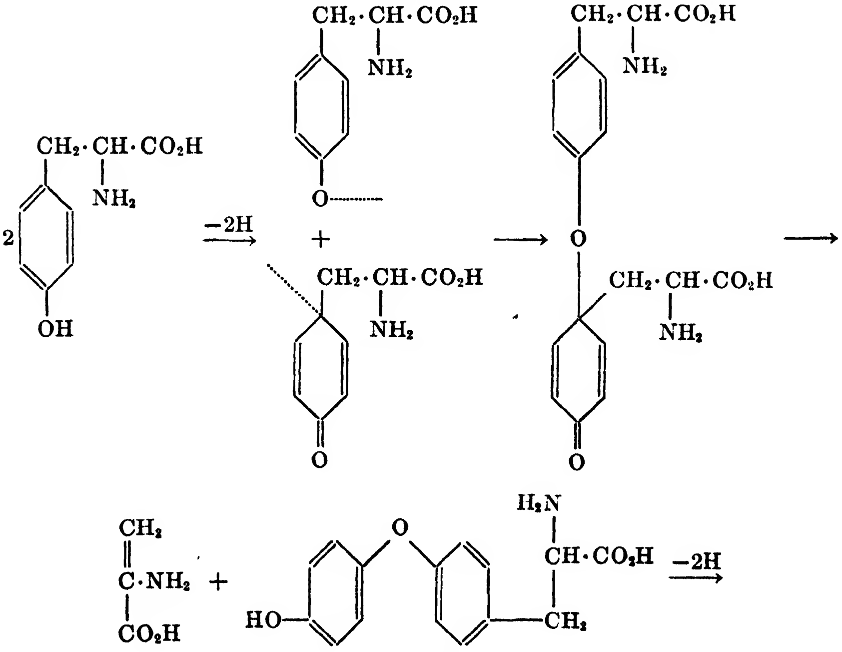
Absorption Curves of Tyrosine and Its Oxidation Product

The points ○ show the per cent absorption for a tyrosine solution. ×, ⊙ and □ show the absorption of solutions of three preparations isolated from mixtures containing three equivalents of cerate per tyrosine molecule. All measurements were made in 0.5 *N* sulfuric acid where the concentration of the substance under investigation was 0.04 mg. per ml.

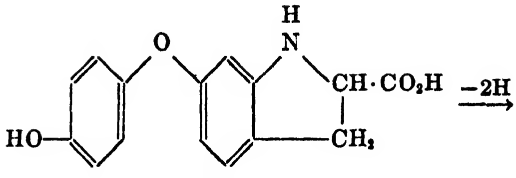
DISCUSSION

The above results offer some indication that an indole derivative was formed when tyrosine was oxidized by ceric salts in strongly acid solutions. It is well known that an indole derivative is formed in the produc-

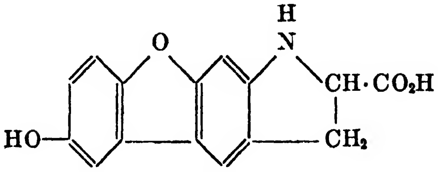
tion of melanin from tyrosine (7). However, since the indole derivative in melanin formation is associated with a blood-red color, and no such color has been observed in this work, the products would appear to be different. A possible explanation is that the indole formation followed a polymerization of the type demonstrated by Pummerer, *et al.* (8, 9) in the oxidation of *p*-cresol by ferricyanide, by various authors (5, 10, 11) in the formation of thyroxine from diiodotyrosine, and by Westerfeld and Lowe (12) in the oxidation of *p*-cresol by peroxidase. Of the above authors only Johnson and Tewkesbury (5) suggest a detailed mechanism for the reaction. In agreement with them we consider it probable that a free radical is formed as the first product of tyrosine oxidation. If two such free radicals dimerized and both side chains formed rings, an easy explanation for the three equivalent oxidation end point would be available. However, the nitrogen content of the compunds isolated suggests the removal of one of the side chains. Such removal would be strictly analogous to the removal of one side chain in thyroxine formation. In this connection it is interesting to note that Felix and Zorn (13) have reported the formation of one mole of alanine from each mole of tyrosine degraded by liver hash. A possible mechanism to explain our results is the following:



(I)



(II)



(III)

The first steps are analogous to those postulated by Johnson and Tewkesbury (5). Compound I or some simple modification of it would account for the nitrogen analyses of our isolated products (found 5.07 per cent; calculated 5.13 per cent), but it would not account for the negative ninhydrin reaction, or, probably, for the absorption spectrum, or for the precipitation with mercuric sulfate under our conditions. These observations can all be explained by an oxidative ring closure to form compound II. This compound explains all the data except the three equivalence of oxidation. It is oxidized only two equivalents per tyrosine, and our data clearly indicate that three equivalents are involved. A possible explanation is the formation of compound III. Another possibility is the oxidative removal of the side chain as Lautenschläger and Bockmühl (14) have suggested for thyroxine formation from diiodotyrosine.

Summary

A potentiometric study of the oxidation of tyrosine, tryptophan, and phenylalanine has been made. Phenylalanine was not oxidized by potassium permanaganate or cerox under any of the conditions tested, but tryptophan and tyrosine were readily oxidized by both oxidants even in 0.5 *N* sulfuric acid. Tryptophan did not show any end point during the addition of four equivalents of either oxidant. Tyrosine showed an end point with three equivalents of cerox. The tyrosine oxidation product was isolated and some of its properties are described. A possible mechanism for the oxidation is suggested.

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A Study of Thiamine Deficiency in the Monkey*
(*Macaca mulatta*)

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Introduction

Since the classical experiments by Eijkman (1, 2) much attention has been given to the various aspects of thiamine deficiency both by clinical and laboratory investigators. Many of the workers have considered the neuropathology, cardiovascular changes, and disturbed enzyme systems, while others have been more concerned with alterations in carbohydrate metabolism, neuromuscular changes, and general symptoms. All of the better known laboratory animals have been used in studying this deficiency disease, but recently observations upon larger animals (3, 4, 5, 6, 7) have given additional information concerning thiamine avitaminosis. Clinical studies on thiamine deficiency have been reported by numerous investigators (8, 9, 10), and several attempts (11, 12) have been made to induce this condition in man through the use of simple diets lacking only this vitamin. An appraisal of the early studies in both animals and man indicates that the observed deficiencies were rarely due to a true thiamine avitaminosis, but more likely were multiple in nature.

Previous reports (13, 14) have clearly shown that the monkey grows

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normally and remains in good health when fed purified diets containing the known vitamins and a small amount of a liver fraction. An uncomplicated thiamine deficiency has been produced by feeding monkeys (*M. mulatta*) a diet in which the liver extract has been treated with sulfite to destroy the thiamine. While thiamine deficiency experiments in the monkey have the advantage that one can observe severely acute changes as well as submarginal states, such studies may not be strictly comparable to certain polyneuritic conditions that are manifest in humans. The present work has dealt primarily with frank thiamine avitaminosis, and should be differentiated from the borderline or suboptimally nourished cases of human thiamine deficiency.

Experimental

The method used for the care of the animals has already been described in detail (13). The basal diet (M-3), consisting of sucrose 73, purified casein 18, mineral salts 4, corn oil 2, and cod liver oil 3, was fed *ad libitum;* and adequate quantities of ascorbic acid, riboflavin, pyridoxin, calcium pantothenate, nicotinic acid, choline chloride, *p*-aminobenzoic acid, and inositol were given daily; one-half cc. halibut liver oil was given once weekly. This diet contained 0.02 γ thiamine per g., thus an intake of from 100 to 200 g. of diet per day would furnish 2 to 4 γ thiamine. In addition to the above vitamins, 2.5 g. of sulfited liver extract (15) were added to the vitamin solution. This rather salty and bitter tasting material was rapidly consumed by the animals until early signs of the deficiency developed. Stomach tube feeding of the sulfited liver and vitamins was resorted to when the animals did not voluntarily drink the entire supplement. In more recent experiments thiamine deficiency was also produced in the animals by replacing the sulfited liver with 20 γ biotin (either a concentrate or the crystalline methyl ester) and a "folic acid" concentrate (16) equivalent to 5 g. of solubilized liver extract. The sulfited liver extract was used in the majority of the experimental diets since it contained insignificant amounts of thiamine in contrast to the 1–2 γ found in each g. equivalent of the "folic acid" preparation.

Thiamine was administered in several ways to the animals during the polyneuritic syndrome: 1) by stomach tube with the daily vitamin supplement, 2) by intraperitoneal injection, or 3) by pipette in the corner of the mouth. During certain periods of this study, pyruvic acid determinations were made on the blood. Electrocardiograms were taken during the deficient stages and after complete recovery.

Experimental Observations

Since the monkeys varied in size, age, and sex, each animal served as its own control in the appraisal of the response to thiamine therapy. This means that the results of pyruvic acid determinations, interpretation of electrocardiogram records, growth effects, etc., were compared to the values obtained in the animal both at the start of the experiment

and during the deficiency. Newly obtained animals placed on the thiamine low diet stopped gaining in from two to eight weeks and animals shifted from adequate diets also declined in about the same period. In all animals a weight plateau was followed by a slight drop which, if allowed to continue for several days, led directly to a sharp loss.

The loss in weight on monkeys on the deficient diet was accompanied by anorexia, a typical finding in most thiamine deficient animals. The decreased food consumption and later the outright refusal to consume the daily vitamin supplement was confirmatory evidence of early acute deficiency. As the disease progressed some of the monkeys showed signs of ptosis. In the later stages of the deficiency many of the animals showed some ataxia and lack of coordination, and an occasional monkey had convulsive seizures followed by a gradual release of the muscle tension. These seizures occurred several times a day and became progressively worse unless thiamine was furnished. If no thiamine were administered, the animal continued to lose weight and could no longer sit on the perch. Foamy exudates from the mouth and nose were visible if the deficiency were allowed to continue for a week to ten days, indicating that cardiac insufficiency may have been part of the syndrome. Unlike deficient pigs (3), thiamine low monkeys did not vomit and in contrast to polyneuritic birds, the deficient monkeys showed no opisthotonus. Rectal body temperatures were taken on the monkeys throughout the latter portion of the study, and no significant temperature changes could be correlated with the extent of the deficiency. The average body temperature remained within a narrow range at approximately 102.3°F.

At autopsy, deficient monkeys showed no striking gross pathology; most organs appeared essentially normal, and the only abnormal findings were congested hemostatic lungs and a slightly greater quantity of fluid in the pericardial sac. No marked differences in heart weight were apparent; no intercurrent infections were observed.

Thiamine Requirements

Twelve animals were given different levels of thiamine to establish the minimum requirement for this vitamin. An attempt was made to bring the animals down to a known weight and then administer daily 10 to 100 γ of the vitamin for given periods, so as to determine the weight gained on a known quantity of thiamine. The number of thiamine feeding trials per monkey varied from 2 to 15 with an average of 5. A part of the data obtained is tabulated in Table I.

The thiamine required for maintaining the monkey at a constant weight is somewhat variable for the individual animals, but is within a narrow range. Approximately 40 γ per day was sufficient for body maintenance. On a daily average intake of 50 γ, a growth response was produced in the majority of the animals, and with 75 to 100 γ of thiamine an optimum response was reached in each monkey. Borderline deficiency symptoms were regularly observed in animals receiving 25 γ or less. Since the monkeys weighed approximately 3 kg., one can conclude from these figures that the minimum (maintenance) requirement

TABLE I

Thiamine Requirement of Individual Monkeys

Monkey No.	Micrograms thiamine per kilogram body weight			Micrograms thiamine per day		
	Insufficient or borderline	Maintenance	Growth	Insufficient or borderline	Maintenance	Growth
52	10.0	16.0	20.0	25	40	50
55	<10.0	16.0	25.0	25	40	>50
58	7.5	14.0	20.0	25–40	40	60
75	<10.0	11.5	16.0	20–30	40	50
93	8.7	18.2	25.0	<50	50	75
107	<12.0	12.0	17.2	25	30	40
108	8.0	10.0	15.0	25	30	40
126	—	23.6	<50.0	—	50	<100
127	—	20.0	27.8	—	40	50
27	<10.0	10.0	—	<50	50	—
28	—	11.2	—	—	40	—
33	—	11.2	—	50	—	—
Ave.......	9.5	14.5	20.8	28	41	52

for thiamine is approximately 15 γ per kg. The optimum requirement for this species of monkey is approximately 25 to 30 γ per kg., which includes the 2 to 4 γ of thiamine furnished by 100 to 200 g. of the basal diet. Within the limits of individual differences in the animals, slightly less than maintenance levels can be used to advantage in producing a chronic thiamine deficiency. This has been accomplished in several animals with symptoms not noticeably different from those observed in acutely deficient monkeys, but more apathy and a greater uncleanliness of the fur and skin were apparent.

The quantity of thiamine in the untreated liver extract (1:20) did not, when fed in small amounts, supply the monkey's optimum thiamine requirement. Monkeys 55 and 127 received the thiamine low diet plus 3% liver extract, and they developed signs of the deficiency more slowly than those animals getting the basal diet alone and no thiamine. From earlier thiamine studies in this laboratory (37) it was known that rats frequently showed mild polyneuritic symptoms on 2% liver extract, while on the 1% liver diet severe convulsions were often observed. Thiamine analyses on the untreated liver extract showed 18 γ of thiamine per g. but the sulfited product showed a barely measurable amount. Thus the decreased food consumption of monkeys 55 and 127 accounted for the inability of the 3% liver extract to provide adequate thiamine. Since these two animals required at least 25 γ per kg. for growth, an intake of as much as 100 g. of the liver diet would be inadequate for a 3 kg. monkey. Monkey 127 weighed less than monkey 55 and therefore the deficiency signs appeared earlier in the heavier animal.

The minimal thiamine requirements of monkeys and other animals may be compared. Van Etten, *et al.* (4) showed the pig's requirement was between 25 and 37 γ per kg. body weight, and Wintrobe, *et al.* (3) obtained somewhat similar values. In a study on the influence of the composition of the diet on the thiamine requirement of the dog, Arnold and Elvehjem (6) pointed out the value of expressing the thiamine requirement as percentage of the diet, and they indicated that the daily requirement for the dogs varied from 13 to 42 γ per kg. of body weight or 75 γ per 100 g. of diet. Fair agreement exists in the thiamine requirement of the large laboratory animals when expressed on a body weight basis. The thiamine requirement of smaller laboratory animals, such as the rat, is less valuable as a basis for comparison with monkeys. When the average maintenance figure for monkeys is used, one can calculate the minimum daily allowance necessary for a 70 kg. man.

Electrocardiographic Studies[1]

More than 67 individual three-lead electrocardiogram (ECG) records were taken at various times on ten of the 29 animals fed thiamine-deficient diets. Each monkey was subjected to the test at least twice, and as many as 16 tracings were taken when the condition of the animal made

[1] Dr. O. S. Orth extended his fullest cooperation in obtaining the electrocardiograms, and Dr. W. J. Meek kindly made the electrocardiograph available for these studies.

it advisable. Ten records were made on the five control animals fed various diets not deficient in thiamine.

The regular leads, I, II, and III were taken through metal clips from the left arm, right arm, and left leg after applying ECG paste well into the fur of the wrists and ankles. The usual method of taking ECG's had to be modified somewhat in studying the cardiovascular changes in the monkey. During the test, the animal was held lying on its side with its arms behind. It was necessary to allow adequate time to elapse before taking the record since the monkeys were somewhat excitable after they were caught.

There were insignificant differences in the tracings when the monkey's arms were held forward or behind, but four of the monkeys, when their arms were held forward, exhibited a slight increase in the height of the R wave and a slight decrease in the heart rate. The contour of the various segments was unchanged by the position of the limbs.

Thirty-five mg. sodium nembutal per kg. body weight were injected intraperitoneally approximately one to two hours before the test, but there were no significant effects on these tracings when compared to those of the same animals unanesthetized.

The quantity of nembutal required to give anesthesia for a given time in thiamine-deficient animals was much less than in animals receiving an optimum amount of the vitamin. While a level of 35 mg. per kg. is ordinarily used, this quantity was toxic to the deficient animals. To illustrate, monkey 90, which weighed 2.23 kg., showed satisfactory anesthesia when it received an intraperitoneal injection of 100 mg. When this animal was very deficient and weighed 1.92 kg., an intraperitoneal injection of 90 mg. of nembutal took immediate effect and resulted in a continually decreasing respiratory rate followed by death within 2 hours.

An analysis of the ECG records (Table II) showed that the heart rate in the thiamine-deficient monkeys was definitely lower than normal. In all of the records no difference was observed in the P-R interval of normal or thiamine-deficient monkeys. The height of the R wave decreased markedly. Another definitive change was the inversion of the T wave in all three leads of several deficient animals, but with administration of thiamine, this wave assumed its upright position (see Fig. 1). Notched QRS complexes occurred in several deficient animals while in others a slight spread of this complex was manifest. Occasional changes in the position of the axis could be attributed to the slightly altered position of the monkey. There was a definite weakening of the myocardium as

TABLE II

Analysis of ECG Records of Thiamine-Deficient and Control Monkeys

Monkey no.	Type of diet	Heart rate		P-R interval		QRS interval		R_{II} wave		T wave	
		Deficient	Optimum	Deficient	Optimum	Deficient	Optimum	Deficient	Optimum	Deficient	Optimum
14	B_1 low	117	168	.09	.08	.03	.04	0.57	.81	Inverted	Upright
52	B_1 low	115	188	.09	.08	.03	.04	0.89	1.22		
55	B_1 low	132	201	.10	.09	.03	.04	1.0	.84		
58	B_1 low	160	214	.10	.08	.03-.05	.04	1.4	1.6	Flattened	Normal
75	B_1 low	130	205	.09	.09	.04	.04	1.0	1.25	Inverted	Upright
90	B_1 low	122	168-188		.08	.03	.03	.4-.6	.80	Inverted	
91	B_1 low	122		.10		.03		.90		Flattened	
92	B_1 low	79	188	.08	.10	.03	.04	.63	.76	Flattened	
93	B_1 low	110	188	.10	.10	.04	.04	1.18	1.46	Inverted	Upright
94	B_1 low	97		.13		.04		.6		Slightly inverted	
57	"Folic acid" deficiency		195		.08		.03		.80		
64	M-3 + 1% liver extract		166		.10		.03		.80		
87	*		150		.10		.04		1.4		
88	*		214		.10		.03		1.0		
63	†		102		.10		.04		.68		
86	†		91		.12		.04		1 60		

* M-3 + "Folic acid" concentrate.

† M-3 + 3% Liver extract + 3 g. sodium salicylate per day.

judged by the height of R wave, together with impairment of normal electrical conduction. Occasional records showed inauguration of the impulse at the sino-auricular node, but reverted to the pacemaker when the vitamin was administered.

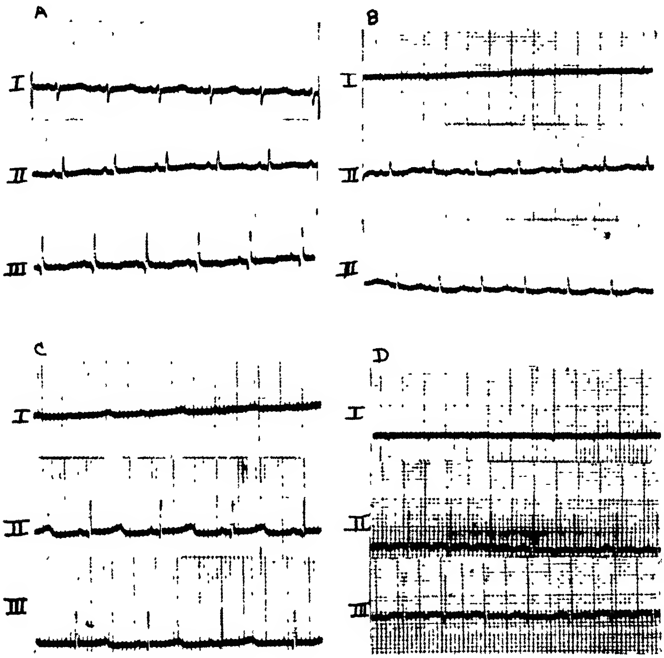

FIG. 1

Typical Electrocardiograph Records of Monkeys Fed a Thiamine-Low Diet

A. Monkey 14—*February 11, 1943.* Thiamine-deficient—Note flattened T_{II}, slow rate and inverted T_{III}.

B. Monkey 14—*May 13, 1943.* Recovered—Note upright T waves and normal rate.

C–D. Stages in thiamine deficiency of Monkey 93. *February 11, 1943:* Note exaggerated T waves. *May 21, 1943:* Note inverted T_{II}, T_{III}.

These findings are in part a confirmation of previous reports dealing with ECG records in other species. The bradycardia observed in these thiamine-deficient monkeys is apparently in disagreement with the tachycardia obtained in dogs by de Soldati (17). Swank, Porter, and Yeomans (5) found elevation of the ST segment in severe deficiency and

"definite abnormalities in the electrical activity of the myocardium and tachycardia just preceding and during periods of cardiac failure." In birds and rats a bradycardia is usually associated with thiamine deficiency (18, 19), but Swank and Beesey (20) found changes in the QRS complex and in the T waves as well as a tachycardia when their pigeons were gradually depleted on a diet containing some thiamine. These workers found bradycardia in deficiency only if there was simultaneous starvation. Wintrobe, *et al.* (21) found a bradycardia, prolonged P-R interval, abnormalities in the P waves, inversion of the T_4, and other changes. These latter workers conclude that in the thiamine-deficient pig, bradycardia is attributable to the deficiency rather than or in addition to inanition. The possible effect of anorexia on our ECG records cannot be denied, but the numerous tracings taken on the individual monkeys during early stages of the deficiency when the animals were still eating, show that the heart rate was already lowered. Slower rates were also obtained in those animals kept on "maintenance" or "borderline" levels of thiamine when the food intake was approximately half that consumed by a normal monkey.

The electrocardiograms of individuals having alcoholic polyneuritis, beriberi, and sub-marginal thiamine deficiency have received a good deal of attention in the clinical literature. Several individual case reports cited by Weiss (8) have given valuable information regarding cardiovascular changes in patients showing either true or artificially induced thiamine deficiency. Goodhart and Jolliffe (22) found inverted T waves and depression of the ST segment together with a slight tachycardia. Swan and Laws (23), and Dustin, *et al.* (24) found a definite tachycardia and inverted T waves which returned to the upright position after thiamine therapy. The studies on induced thiamine deficiency by Williams, *et al.* (12) have shown a bradycardia at rest and a tachycardia after exercise. Weiss (8) and his associates have observed a tachycardia and an inversion of the T waves.

It was felt that two monkeys Nos. 63 and 86, which received the 3% liver extract diet plus 3 g. sodium salicylate per day could serve as two of the controls for electrocardiographic studies, but the ECG records during this period indicated a definite decrease from a normal heart rate of approximately 200 to 91–102 beats per minute. When the salicylate was discontinued, the heart rate gradually returned to normal. Hanzlik (25) has cited the work of several investigators who noted a definite slowing of the pulse when salicylates were administered to various

animals. Monkeys fed diets adequate in thiamine served as additional controls and are also listed in Table II.

Blood Pyruvic Acid Studies

These determinations were made according to the method of Bueding and Wortis (26) on blood drawn from the saphenous vein of the leg. Blood samples were obtained several times during the incipient and acute stages of the deficiency and compared to the pyruvic acid values obtained after the monkey had been given thiamine. Although it is recognized that this method does not differentiate between the various carbonyl compounds in the blood, the "pyruvic acid" determinations have significance when comparisons are made in the same animal at various stages of the experiment. On a relative basis then, the pyruvic acid levels in the blood might give additional information as to the extent of the deficiency.

Table III shows average pyruvic acid levels in monkeys before the onset of thiamine deficiency, and during the acute stage of polyneuritis. The pyruvic acid level in the blood during deficiency is 2–3 times greater than that found in the normal animal although several values ranged as high as 13.2 mg.%. The normal levels of pyruvic acid listed in the table are somewhat higher than those of Wintrobe (3) and definitely higher than that reported for humans (12, 26).

In order to test the effect of decreased food intake on the level of pyruvic acid, several of the animals were starved for 36 hours before a blood sample was taken, but this period of food deprivation had no effect on the level of carbonyl compounds in the blood. In several of the animals, the pyruvic acid value remained elevated after borderline amounts of thiamine were fed in spite of the better physical condition which prevailed, but when optimum amounts of thiamine were given, the value dropped rapidly. Conversely, it was found that the low pyruvic acid level in monkeys rose sharply when the thiamine was discontinued. Rapid changes in the pyruvic acid level were therefore a reflection of the narrow range between deficiency or maintenance and optimum conditions in these animals.

Whether pyruvic acid plays any rôle in causing the physical signs of the deficiency is not definitely established. While the work of Nitzescu and Angelescu (27) would indicate that pyruvic acid and lactic acid are the causative agents of the opisthotonus in pigeons, carbonyl compounds *per se* are of dubious significance in producing neurological

symptoms in man, and probably reflect only a disturbed carbohydrate metabolism. Monkey 55 received borderline amounts of thiamine per day and had outward signs of polyneuritis, although the pyruvic acid determination on the blood showed only 2.0 mg.%; at a later period of acute deficiency this same monkey had a level of 9.5 mg.%. It may be that during the earlier period, the level of thiamine was insufficient for

TABLE III

Pyruvic Acid Levels in Blood of Thiamine-Deficient and Control Monkeys

Monkey no.	Before deficiency *mg. %*	During deficiency *mg. %*	Extent of deficiency
27	2.7	6.3	++
28	2.6	4.6	+
33	2.4	6.1	+
52	4.6	9.2	++
55	2.0	9.5	++
58	<5.4	7.2	++
75	4.0	9.9	++
93	3.5	6.9	+
107	3.9	7.7	+
108	3.7	7.4	++
126	3.4	—	
127	3.6	5.7	+
26	2.6	—	
32	2.6	—	
53	2.7	—	
63	2.5	—	
79	2.5	—	
82	4.1	—	Riboflavin-deficient
89	3.3	—	
131	3.2	—	Received 3 g. Na pyruvate/day
132	4.2	—	as above
133	3.4	—	as above
134	2.9	—	as above
Ave.	3.2	7.3	

saturation of the tissues, but was enough to give normal carbohydrate metabolism. Other monkeys gave similar indications, especially when the period of depletion was longer than the average time required to produce the deficiency.

A number of "metabolic load tests" (3) were performed on both thiamine-deficient and normal monkeys. On the morning of the test, the monkeys received no food; samples were drawn at half hour intervals

for two to three hours; immediately after the first sample was taken, 1.75 g. glucose per kg. were given by stomach tube. In several of the deficient monkeys, the level of pyruvic acid in the blood rose to a peak within ½ to 1 hour, and then fell to the original level. In other thiamine-low animals, a very high pyruvic acid level (12–14 mg.%) was obtained, but after the glucose feeding this value dropped steadily and reached a normal level (2–4 mg.%) at the end of three hours. While Bueding, Stein, and Wortis (28) found elevated values for pyruvic acid in blood after oral administration of dextrose to thiamine-deficient patients, Williams, *et al.* (3) found curves of similar contour, but raised at all levels after intravenous injections of glucose. A correlation of the extent of thiamine deficiency in monkeys with the results of the metabolic load tests does not furnish any explanation for the continued drop of the pyruvic acid to the normal level three hours after the tube feeding of glucose.

Four animals received 4 g. sodium pyruvate (29) per day, and their blood pyruvic acid values determined both before eating and in the post-absorptive state showed no increase over that observed in animals not fed added pyruvate. This is confirmation of previous work in chicks (30) that either parenteral or injected pyruvate is rapidly destroyed and increased blood levels of this substance cannot be demonstrated.

Discussion

The symptoms of thiamine deficiency in the monkey are similar to those observed in other animals, except that the monkey was free from signs of opisthotonus and vomiting. Generalized muscular weakness existed, with ptosis, loss of the grasping reflex, and convulsive seizures. The body temperature of the deficient animals remained essentially normal, and red cell, white cell, and hemoglobin determinations were also normal.

A comparison of the thiamine deficiency signs observed in our monkeys with those described by Leblond and Chaulin-Serviniere (31) in spontaneous beriberi of colony monkeys shows substantial agreement. The unsteady gait and the neuromuscular disorders followed by a terminal phase of acute dyspneic symptoms observed by these workers were also seen in our animals maintained in a chronic state of thiamine deficiency. In their animals fed a poor colony diet definite cardiac disturbances and degeneration of the myelin sheath in nerves of involved limbs were demonstrated. The lack of several dietary factors in both the

experimental and colony diets used by Leblond and Chaulin-Serviniere may have partly accounted for the nerve degeneration (3). The pathological changes in the nerves can also be explained now by the work of Street, *et al.* (7) who found that a "prolonged subminimal intake of vitamin B_1 with an otherwise adequate diet leads to degenerative changes both of the peripheral nerves and of the posterior columns of the spinal cord. This would appear to be chiefly due to the lack of vitamin B_1 in the tissues, rather than to the inanition accompanying the deficiency state." Swank and Bessey (12, 32) found that a chronic deficiency in pigeons resulted in degeneration of the peripheral nerve, but if thiamine therapy was instituted before neuron death, recovery of the nerve was attained. Adequate controls excluded the effects of inanition or absence of other factors. Follis, *et al.* (33) found no changes whatever in the nervous systems of thiamine-deficient pigs. Nervous and cardiac disturbances in a monkey given a diet of unwashed polished rice were reported by Shiga and Kusama (34) while in McCarrison's experiments (35) in which an autoclaved rice diet was used, the monkeys died rapidly with no outstanding symptoms. These last two studies were undoubtedly complicated by multiple deficiencies, although symptoms due to the lack of thiamine were observed first.

The increased "pyruvic acid" levels in the blood show that the disturbed carbohydrate metabolism in thiamine deficiency exists quite apart from any complicating starvation effects. The increased "pyruvic acid" levels in the animals which were still eating well rule out anorexia as a troublesome influence in these experiments.

The relation of the deficiency to the condition of the heart has been the subject of many clinical and laboratory investigations, but no acceptable explanation is available as to the exact mechanism by which the heart musculature is affected. Thomas, *et al.* (36) found that severe myocardial lesions are dependent upon the combined deficiency of potassium and several of the B vitamins. The electrocardiographic studies reported here confirm the findings in other thiamine-deficient animals, but the knowledge that the T waves are inverted, or that the rate is slowed or that the P-R interval is unchanged does not explain the precise rôle of the vitamin. The accumulation of carbonyl compounds resulting from disturbed carbohydrate metabolism may actually be poisoning the normal system of nerve impulse transmission as has been suggested by other workers.

A recent report by Najjar and Holt (38) described the production of

thiamine deficiency in 4 of 9 patients fed a synthetic diet. One of the symptom-free subjects was given succinyl sulfathiazole, and a sharp drop in the fecal thiamine was noted. The absorption of thiamine from the large intestine was demonstrated in the patients indicating that intestinal bacteria supply some thiamine to man. Since all of our monkeys showed satisfactory signs of polyneuritis, it is possible that intestinal synthesis is of negligible influence on the course of thiamine deficiency in the monkey. Biosynthesis, however, has been shown to be a prominent factor in other deficiencies in this animal (39).

Summary

1. Thiamine-deficient monkeys show a drop in weight, decreased food consumption, general muscular weakness, loss of reflexes, convulsions, incoordination, increasing cachexia, signs of cardiac insufficiency, prostration, and death. No vomiting and opisthotonus were observed in the 29 animals studied for periods of from 2 to nearly 13 months.

2. The minimum thiamine requirement for maintenance of monkeys weighing about 3 kg. is 15γ per kg. of body weight or approximately 40γ per day. The minimum requirement for growth is between 25 and 30γ per kg. per day, or a total of about 75–100γ per day.

3. A distinct lowering of the heart rate has been recorded through electrocardiographic studies uncomplicated by the influence of decreased food intake. A definite decrease in the height of the R wave and inversion of the T wave were observed with no change in the P-R interval.

4. The "pyruvic acid" level in the blood of normal monkeys is higher than the corresponding figure for the pig and man, but in thiamine-deficient monkeys it is sharply increased.

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Manganese Deficiency in the Rabbit

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Introduction

The literature on manganese deficiency in the rat and mouse has recently been reviewed and well summarized by Shils and McCollum (1943) and Wachtel, Elvehjem, and Hart (1943), and in the chick by Wilgus, Norris, and Heuser (1939). In addition to the above mentioned species, evidence has been presented that lameness and crooked legs occurring in pigs on an experimental diet is due to manganese deficiency (Miller, Keith, McCarty, and Thorp, 1940; and Keith, Miller, Thorp, and McCarty, 1942).

In the course of studies of milk anemia in rabbits, it was noted that a large number of animals developed severely deformed front legs. Further studies showed that this condition was due to a deficiency of manganese, and the results are presented here. The deformation of leg bones of rabbits fed an exclusive milk diet has previously been noted by Brouwer (1926) although the etiology was unknown.

Preliminary Studies

Crooked front legs were first noticed in a group of 19 rabbits that were being fed cow's milk enriched with 10 per cent of whole milk powder. Among the 12 rabbits living at 10 weeks of age, 8 showed either a splaying or a bowing of the front legs. A second preliminary experiment was started with 24 rabbits of which 12 received only the milk enriched with milk powder diet. Each of the remaining 12 received, in addition, 2 mg. of Mn per day. Of the 12 which received only the milk diet, 9 developed severe crookedness of the front legs at 6–10 weeks of age; whereas of those which received the Mn supplement, 3 developed moderate and 4 slight to questionable crooked front legs. This experiment indicated

that Mn was involved but that 2 mg. per day was insufficient. Consequently, a third experiment was initiated which is here reported in detail.

Materials and Methods

Rabbits from our colony of the Dutch breed were placed on a milk diet in a manner similar to that used by Elvehjem and Kemmerer (1931) to develop milk anemia in rats. The rabbits were weaned at 4 weeks of age and placed in individual wire-bottomed cages. In the preliminary studies, milk plus 10 per cent of whole milk powder[1] was used, but it was decided to make a slight change and feed only whole milk powder inasmuch as it is more convenient, and it was thought that greater growth would result from the increased solids intake. Eight rabbits (5 females and 3 males) were fed a basal diet of milk powder ad libitum and a supplement of 2 mg. of iron, 0.2 mg. of copper, and 0.1 mg. of pyridoxin per day. Seven rabbits (5 females and 2 males) were fed the above diet but in addition were given 5 mg. of Mn per rabbit per day which was administered by mouth twice weekly. The iron was fed as ferric chloride which was prepared from carbonyl iron and redistilled 1:1 hydrochloric acid. The carbonyl iron was obtained from the General Aniline Works, Grasselli, New Jersey. Copper was fed as cupric nitrate which was prepared from electrolytic copper and redistilled 1:1 nitric acid. The source of manganese was manganous chloride, c.p., dissolved in redistilled water. The pyridoxin was included since unpublished data indicate that a milk diet may be deficient in this vitamin for the rabbit. The rabbits were weighed weekly and carefully observed for abnormalities. Two rabbits, one from each group, died early in the experiment from causes that could not be ascertained and are not included in the following discussion. In the group not fed Mn, three animals died at 12, 15, and 17 weeks of age, respectively. The experiment was terminated when the animals were 18 weeks of age.

At autopsy the right radius, ulna, and humerus were removed, dissected free of tissue and air dried. Bone volume and density were determined on the air dried bones using the technique of water displacement. The length and minimum shaft diameter of the humeri were determined with a micrometer caliper. The breaking strength of the ulnae was estimated by running water into a bucket suspended from the bones placed between two set supports. These supports were placed 2.5 cm. apart and were made of steel rods 6 mm. in diameter. The weight of the bucket and water at the time of breaking gave the breaking strength. Care was taken to place each bone in as nearly comparable a position as possible. The humeri were then dried at 100°C., extracted with ether, and the ash content determined. The entire livers were removed, and the manganese content determined using the method of Skinner and Peterson (1930) with the exception that the transmission of the final solution was determined in a photoelectric colorimeter using a 5 cm. absorption tube. The ovaries, testes, and heads of the left humeri were removed, fixed, embedded, sectioned, and stained for microscopic study.

Results

All of the rabbits which received the Mn supplement had normal appearing legs at the end of the experiment, and they were in excellent

[1] Borden Company, New York City. Prepared in all stainless steel equipment.

condition although growth was only about 60 per cent that of comparable rabbits fed a stock diet. Six of the 7 rabbits which received the basal diet unsupplemented with Mn developed gross deformities of the front legs which were first noted at 7–14 weeks of age. Although the diet of whole

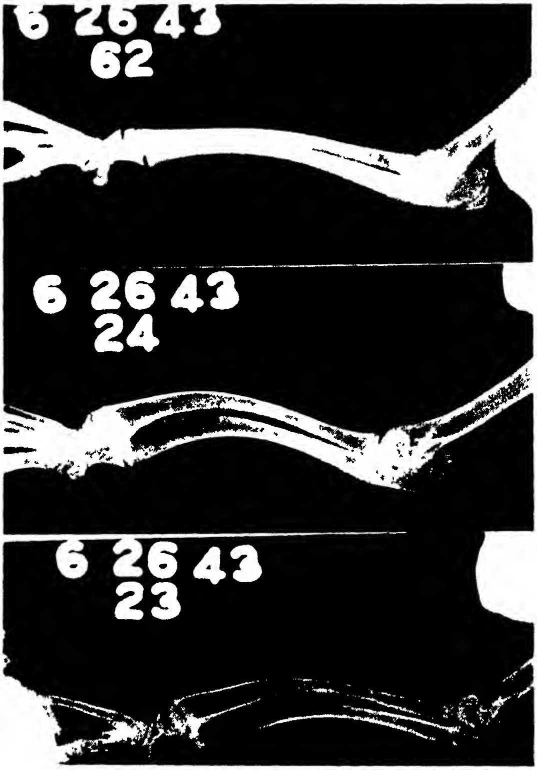

Fig. 1

X-Ray Pictures of the Forelegs of a Control Rabbit (Top) and Mn-deficient Rabbits in which Bending of the Radius and Ulna and Decalcification are Evident

Note also the enlargement of the head and shoulder of the distal end of the radius and ulna

milk powder did produce greater growth than the diet of milk enriched with milk powder used in the preliminary experiments, the onset of crooked legs was delayed, and the crookedness was less severe. The dissected bones of the front legs showed that the bowing was confined to the radius and ulna (Fig. 1). Deformity of the hind legs has not been

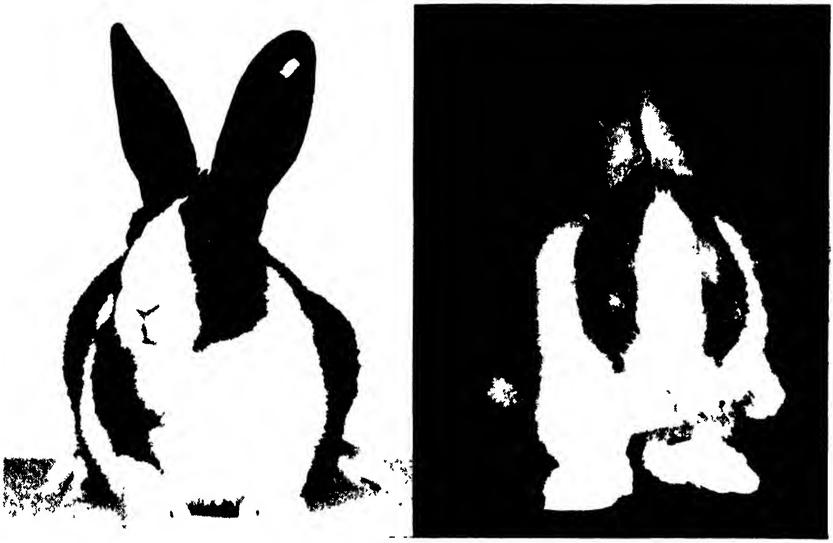

FIG. 2
Pictures of a Control Rabbit (Left) and a Mn-Deficient Rabbit (Right) Showing the Gross Deformity of the Front Legs

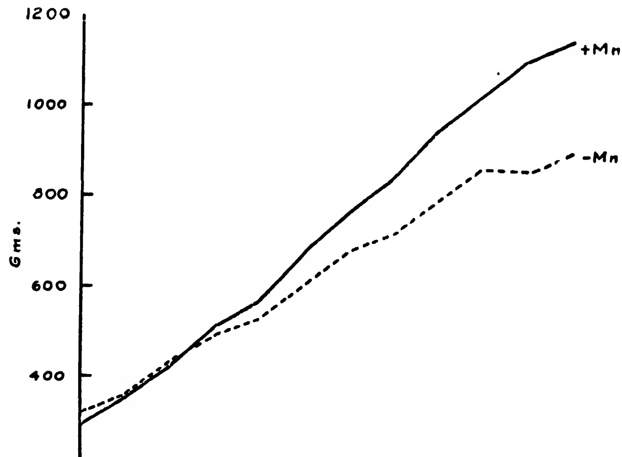

FIG. 3
Composite Growth Curves of Control Rabbits (6) and Mn-Deficient Rabbits (6)

noted. The gross appearance of the front legs in the living animal (Fig. 2) showed a greater deformity than can be accounted for by the radius and ulna. It appears likely that in the living animal a weakness of the carpus exaggerates the bone deformity.

Fig. 3 presents the growth curves of the two groups which include all rabbits living at 15 weeks of age. It will be noted that the growth of the Mn-deficient group is significantly less than that of the Mn-supplemented group. This observation is in agreement with those of others made on the rat, mouse, and chick.

TABLE I

Weight, Volume, Density, Length, Diameter, Breaking Strength and Ash Content of the Bones and the Manganese Content of the Livers of Control and Manganese-Deficient Rabbits

	Basal diet plus Mn*	Basal Diet**	Significance
Weight of dry, fat-free humeri (g.)	1.205 ±0.0411	0.923 ±0.1020	Significant
Volume of humeri (ml.)	1.587 ±0.0732	1.449 ±0.1192	None
Density of humeri (g./ml.)	1.040 ±0.0151	0.818 ±0.0562	Highly significant
Length of humeri (mm.)	59.70 ±0.853	52.63 ±2.113	Highly significant
Diameter of humeri shaft (mm.)	3.78 ±0.062	3.79 ±0.129	None
Breaking strength of ulnae (lbs.)	13.54 ±0.994	9.04 ±1.490	Significant
Ash in dry, fat-free humeri (%)	61.375 ±0.4686	55.669 ±0.9811	Highly significant
Manganese in liver (γ/g. fresh liver)	2.50 ±0.056	0.51 ±0.049	Highly significant

* Mean values of six animals.

** Mean values of seven animals.

A summary of the weights of the dry, fat-free humeri, volume of humeri, density of humeri, length of humeri, minimum diameter of humeri shafts, breaking strength of ulnae, percentage of ash in the dry, fat-free humeri, and the manganese content of the livers is presented in Table I. The values given are means with their standard error. The significance of mean differences was determined by a t test. P values of

0.05 are arbitrarily considered as significant, and P values of 0.01 are considered highly significant.

It is noted that the weight, density, length, breaking strength, and ash of the bones are less in the Mn-deficient than in the Mn-supplemented rabbits. The volume of the humeri and minimum diameter of the humeri shaft do not differ significantly between the two groups. The humeri are thus shorter and thicker. The increased thickness of the humeri is evident in the head and shoulder as noted in Fig. 1. The decreased breaking strength of the bones of Mn-deficient rabbits has been frequently noticed at autopsy. The ribs were particularly brittle and readily broke under slight flexing. In the preliminary experiments, a spontaneous fracture of the spinal column occurred in three rabbits which produced a posterior paralysis. Although the bone ash content is significantly decreased in the Mn-deficient rabbits, the decrease is much less than that which occurs in rickets. Other evidence that this bone abnormality is unlike rickets is found in X-ray pictures of the legs where the epiphyseal line is regular and shows no significant cupping as found in rachitic animals. The manganese content of the livers is distinctly lower in the Mn-deficient rabbits being only about 20 per cent of that of the Mn-supplemented rabbits.

At the end of the experiment, hemoglobin was determined on each animal using the method of Sanford and Sheard (1930). The Mn-deficient rabbits ranged from 10.2–12.4, and the Mn-supplemented rabbits 11.6–13.2 g. hemoglobin per 100 ml. of blood. The numbers of observations are too small to show whether or not there was a significant decrease in hemoglobin. Wachtel, Elvehjem, and Hart have shown that Mn-deficient rats tend to run slightly lower in hemoglobin than normal rats.

A microscopic study of the heads of the humeri has shown extensive alterations in the Mn-deficient rabbits, which differ distinctly from the changes found in rachitic bones. The most evident change is a narrowing of the zone of provisional calcification due to an appreciable reduction in the number of cartilage spicules. There also appears to be a delay in the laying down of new bone on the spicules that do exist. As a result of the reduction of cartilage spicules, the epiphyseal plate is narrow and sharply defined, and there is a deficiency of spongy bone (Figs. 4 and 5). No evidence of accelerated bone resorption was found. The over-all picture suggests a suppression of osteogenesis. Wachtel, Elvehjem, and Hart mentioned that in the Mn-deficient rat, longitudinal sections of the bones showed an absence of trabeculae in the head of the diaphysis.

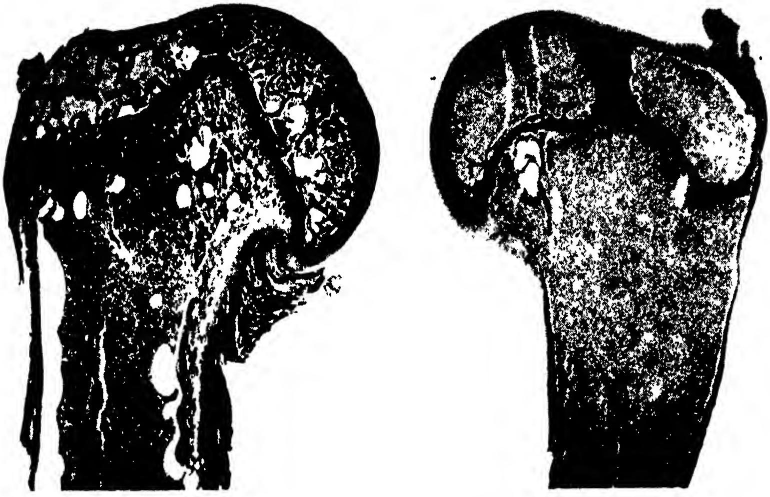

Fig. 4

Low-power Photomicrographs of Humeri Taken from a Control (Left) and a Mn-Deficient Rabbit (Right)

Note the deficiency of spongy bone in the Mn-deficient humerus and the thin compact bone of the shaft

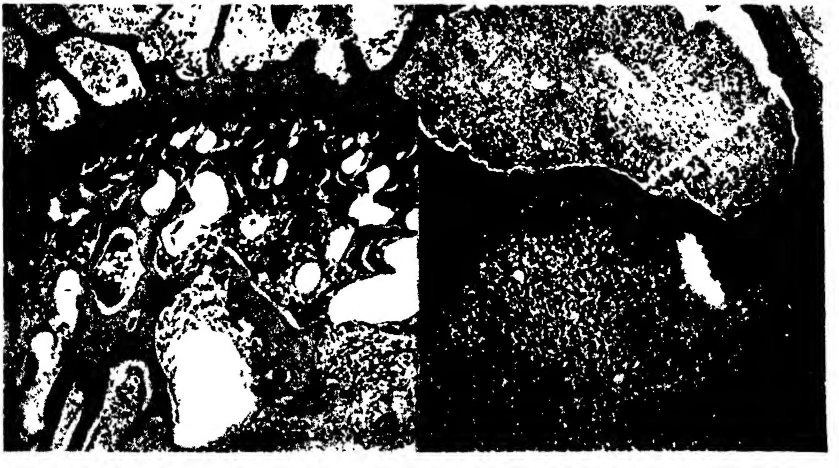

Fig. 5

High-Power Photomicrographs of the Humeri of Fig. 4 Taken in the Area of the Epiphyseal Plate Showing an Extreme Narrowing of the Zone of Provisional Calcification in the Mn-Deficient Humeri (Right)

The deficiency of spongy bone is very evident in this bone which was the most extreme case found

Although there was a significant decrease in the thickness of the compact bone of the shaft in Mn-deficient rabbits as compared to the controls, such bone was dense and gave no evidence of abnormal resorption.

The bone marrow of the Mn-deficient rabbits appeared to be more cellular than the controls, but the significance of this is not known. If there is a tendency in manganese deficiency to suppress hemoglobin formation as suggested in this study and shown in the rat by Wachtel, Elvehjem, and Hart, the increased cellularity may reflect the effort of the bone marrow of the long bones to compensate for this deficiency.

A microscopic study was also made of the gonads, but unfortunately the number (3) of males in the deficiency group was too small to permit conclusive statements. The testis of one male showed extensive tubular degeneration. In the extreme, the degenerate tubules contained only a single row of cells lying next to the basement membrane and consisting of a few spermatogonia and Sertoli cells. The remainder of the cells in the tubules apparently were sloughed off and excreted or absorbed. The lumina of the epididymis were filled with degenerating cells. This testis was not uniformly degenerate for severely degenerate bundles of tubules were interspersed among normal appearing tubules. Mason (1926) has pointed out that in vitamin E deficiency some tubules are apparently more resistant to degeneration than others. The testes of the other two males gave evidence of beginning degeneration in that many multinucleate giant cells were present in the tubules and the more mature forms of cells (spermatids) were starting to slough off. Superficially, at least, this testis degeneration is similar to that of vitamin E deficiency in the rat which in turn is similar to that caused by a number of conditions—inanition, alcoholization, X-ray treatment, and others (Mason, 1926, 1933).

Although the ovaries and uteri of the female rabbits were found to be significantly smaller than the controls, microscopic study did not reveal any significant alterations except possibly that more degenerating follicles, mostly secondary follicles, were noted in the deficient females.

Discussion

Heretofore distinct malformation of the skeletal system as a symptom of manganese deficiency has been described only in the chick, although some evidence of bone deformities has been presented for other species. Barnes, Sperling, and Maynard (1941) found two cases of abnormal tibias out of 16 rats raised on a Mn-deficient diet. Miller, *et al.* (1940)

mentioned enlarged hock joints and crooked legs in pigs, although this symptom was not emphasized. In the recent paper by Shils and McCollum (1943), it is mentioned that "surviving young of markedly deficient females develop skeletal abnormalities. The gross effect is a shortening and bowing of the forelegs. The pathological changes will be described elsewhere in detail." This brief description appears to be much like that observed in rabbits. A point of difference, however, is that in rabbits, the deformed legs appear in the first generation of animals placed on experimental diets; whereas according to the above statement, it occurred in rats only in the offspring of deficient females.

Summary

Manganese deficiency in the rabbit seriously interferes with normal bone development and is grossly most evident in severely deformed front legs. There is a decrease in breaking strength, weight, density, length, and ash content of the humeri of deficient animals. A microscopic study of the humeri revealed extensive deviations from normal which are interpreted as suppressed osteogenesis.

There was a significant decrease in growth and a decreased content of manganese in the liver of Mn-deficient rabbits as compared to controls. Testicular degeneration was found in the deficient males.

The authors are grateful to Dr. P. Olafson, Professor Veterinary Pathology, Cornell University, for aid in the microscopic studies.

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Growth Factor Requirements of Three Saccharolytic Butyl Alcohol Acetone Bacteria

Lampen and Peterson (1943)[1] found that biotin was essential for the growth of 20 organisms of the *Clostridium* group and that *p*-aminobenzoic acid, in addition to biotin, was required by members of the *Cl. acetobutylicum* type.

Studies on the growth factor requirements of three strains of saccharolytic butyl alcohol-acetone bacteria have been made. Two of these, which we have tentatively named B_1 and B_2, were found to grow in a synthetic medium with biotin only, while the third, BA_1, required in addition *p*-aminobenzoic acid.

The basal medium contained 3% glucose, 0.3% ammonium sulfate, Speakman's salts and a little calcium carbonate. Biotin, in the form of the hydrolyzed methyl ester, and *p*-aminobenzoic acid, were added in amounts of 0.001 μg. and 0.2 μg. per ml. of medium, respectively.

To prepare the inoculum, a tube of freshly boiled potato mash was inoculated with a loopful of the stock culture in soil. After 24 hours' incubation, a transfer was made into a medium containing 2.55% glucose and 0.45% molasses. This glucose-molasses culture was centrifuged at the end of 24 hours, and the cells were suspended in sterile distilled water, centrifuged and resuspended in sterile water. One-tenth ml. of this preparation was used to inoculate 10 ml. of medium.

The amount of growth was measured by the quantities of sugar left after incubation under anaerobic conditions. The following table shows the residual sugar in the various media in which the three cultures were tested.

Culture	Residual sugar, in percentage, after 7 days			
	B.M.* only	B.M. + Biotin	B.M. + P.A.B.	B.M. + Biotin + P.A.B.
B_1	2.85	0	3.13	0.2
B_2	2.87	0	2.96	0.2
BA_1	2.91	2.89	2.96	0.86

* Basal medium, control.

[1] Lampen and Peterson, *Arch. Biochem.* **2**, 443 (Aug. 1943).

It will be noted that the three organisms were unable to utilize the sugar in the basal medium alone, and in that containing only *p*-aminobenzoic acid. In the presence of biotin, however, B_1 and B_2 consumed all the available sugar. BA_1 showed active fermentation only when both biotin and *p*-aminobenzoic acid were present.

Six other known growth factors—thiamin, riboflavin, pyridoxin, nicotinamide, inositol and pantothenic acid—had no effect on the growth of the cultures.

Hygienic Laboratory
University of Michigan
Ann Arbor, Michigan
April, 1944

R. Reyes-Teodoro
M. N. Mickelson

BOOK REVIEWS

Laboratory Manual of Biochemistry. By BENJAMIN HARROW, GILBERT C. H. STONE, ERNEST BOREK, HARRY WAGREICH and ABRAHAM MAZUR of the Chemistry Department, City College, College of the City of New York. Second Edition, Revised. W. B. Saunders Company, Philadelphia and London. 1944. V + 132 pp. Price $1.50.

This revision of a manual which appeared in 1940 is designed to furnish the laboratory work for a course in animal biochemistry. The work includes the study of the qualitative properties of carbohydrates, lipids, proteins and enzymes, together with such fluids as saliva, milk, gastric and pancreatic juices, bile and blood. Quantitative methods for the estimation of the more important constituents of blood and of urine are an important feature.

The directions are simple and precise, they appear to be the result of considerable experience in teaching. The questions following each experiment will be of value in arousing the student to carry over the information from his classroom work into the laboratory. The principles and manipulative details for any unusual apparatus are fully described. Among the less common experiments there are included: a chromatographic analysis of the pigments of a plant extract; the enzymatic determination of urea by the manometric method; the estimation of a sulfa drug in blood; and the estimation of bilirubin in serum. If the reviewer were to point to any field which could be included we would suggest work on the more common minerals in food and blood, together with studies on bones and teeth. The format is good and the metal ring binding makes the manual more adaptable to use in the laboratory.

W. M. SANDSTROM, Minneapolis, Minn.

Biochemistry of Disease. By MEYER BODANSKY, and OSCAR BODANSKY. The MacMillan Co., New York, N. Y., 1942. viii + 684 pp. Price $8.00.

In the last few decades clinical medicine has become to a large extent applied biochemistry. This synthesis between clinical medicine and theoretical biochemistry is only part of a general trend toward closer relations of the pre-clinical branches of medicine in its functional aspects, such as physiology, biochemistry and pharmacology, with medical practice at the bedside. The book "Biochemistry of Disease" by M. and O. Bodansky, which was first published in 1930, is one of the first attempts undertaken "with the conviction that a systematic presentation of the biochemical aspects of the various diseases, arranged according to clinical entities would be of distinct value and use to the physician." A revised edition of the book appeared in 1937 followed by reprints in 1938 and—in the form of the present edition—in 1942.

Notwithstanding this outstanding success the reviewer feels bound to express a few critical remarks. In the first place the arrangement according to clinical entities has its definite disadvantages, such as necessary repetitions (for instance renal rickets is treated as a disease of the kidneys as well as that of the bones), omission of more general but still practically important biochemical research items, including biochemistry of malignancy or of sulfonamide effect and discussion of diseases without available pertinent biochemical data. Objection should also be raised to reprinting a book on biochemistry without any revision in the last five years. The progress in such a period was too great and in consequence the last edition is not "up to date" when published.

In spite of these critical remarks to which more could be added, full credit should be given to the authors not only for their excellent work in compiling all the information used, but mainly for their attempt to bring about a visible junction between biochemistry and clinical medicine.

PAUL GYÖRGY, Cleveland, Ohio.

The Biochemistry of Malignant Tumors. BY KURT STERN, M.D., formerly Research Associate of the University of Vienna, New York, N. Y., AND ROBERT WILLHEIM, M.D., Professor, University of the Philippines, Manila. Reference Press, Brooklyn, N. Y., 1943. $12.00.

About 30 years ago, it became quite manifest that the problems of cancer and of the malignancy of tumors cannot be solved by morphological studies alone. Thus, the investigation turned toward biochemical studies, and the microscope was gradually replaced by the test tube of the chemist in cancer research. This was at first done timidly, later more boldly, and finally the biochemistry of cancer developed into a vast domain of research with thousands of publications dealing with the questions involved.

Two methods of study appear promising: the chemical study of cancerous tissue and the cancer cell, and the study of the metabolism of the organism of the tumor host, including clinical and laboratory findings in the human cancer patient. Experimental methods were widely adopted for these studies.

The book of Stern and Willheim represents an attempt to collect the present-day material on the biochemistry of cancer into a summary which must "satisfy the expert in the field, as well as the reader who is only generally interested in biology or medicine." The tendency of the book is "to cover the relationship of cancer to chemistry in the broadest meaning of both words, based on a collection of the literature as complete as possible." The book is a revised edition of that published in German in 1936. The English edition takes into consideration about 5000 references to the literature, which had been followed until the first half of 1942.

The contents of the book are arranged in the following order: "Inorganic Chemistry," "Organic Chemistry" (including chapters dealing with "Organic Carcinogenic Substances" and "Organic Chemotherapy of Tumors"), "Physical Chemistry" (including "Biophysics"), "Enzymes," "Nutrition and Vitamins," (including a chapter on "Dietary Treatment of Human Cancer"), "Metabolism," "Endocrine Glands and their Hormones", "Immunology" (including chapters on

"Specific" and "Unspecific Immunotherapy of Neoplasms"), "Biochemical Aspects of Tumor Origin and Tumor Growth" (including chapters about "Heredity and Extra-Chromosomal Factors" as well as about "Virus Tumors") and "Chemical and Biological Tumor Diagnostics."

Thus, in addition to the biochemical problems, a great many topics are discussed which may only be related indirectly to chemistry, changing the book into a review of the world literature, covering the whole field of cancer theory from the viewpoint of the biochemist.

In the chemical chapters of the book analytic data are reported concerning the occurrence of the discussed substance in the tumor tissue as well as in the tumor host. Further, the effects of the substance on the appearance, growth, and metabolism of tumors are described. The possibilities of any significance of the substance to the problem of malignancy are discussed, and attempts to influence animal and human cancer by the substance are considered, when undertaken. Extensive Author and Subject indices guide the reader to the pertinent references.

The often conflicting, or even completely contradictory, findings reported in the literature are reviewed in a concise and impersonal manner, rarely introducing critical points of view. This is done purposely, and is openly admitted in the preface. In this way a practical source of basic orientation in the actual state of the discussed questions may be found in this book, free from suggestive interpretations of the recounted facts.

On the other hand, the general impression thus created concerning the present-day status of our biochemical knowledge of cancer becomes quite depressing for the non-expert. He will easily agree with the authors that "proven findings of characteristic nature, distinguishing malignant tumors from the norm or from other pathologies, are but few, and even here it is still debatable whether, and to what extent, these data are connected primarily with the cancerous process, or instead represent only secondary changes," including phenomena of tissue decomposition and general cachexia of the tumor host.

The specialist knows that important achievements have been made in the biochemistry of cancer. But today they remain loose bricks only, waiting for the creative genius who will connect them into the edifice of a working cancer theory.

The biological and medical problems discussed in the book include an extensive review of the literature, reporting diagnostic, etiologic and therapeutic implications. The review shows that "there is a mass of findings the value of which is not in proportion to the work done".

Here again few decisive statements may be found, even when the authors discuss the role of the reticulo-endothelial system in cancer, to which they seem to ascribe special importance. Every better-known theory is reported and its real and hypothetical basis is described. Among others, the important investigations of E. Freund are given, a very fortunate circumstance in view of the fact that, owing to language difficulties, they have been somewhat neglected in the English literature.

The cytolysis of cancer cells and its significance for cancer diagnosis, a field of research in which the authors have made many contributions, also receives an adequate place in the chapter dealing with tumor diagnosis.

Summarizing, the book of Stern and Willheim is a very useful reference volume for everyone looking for first aid when confronted with the need for a rapid orientation in the literature of cancer problems.

The specialist, as well as the interested layman, including the doctor treating cancer patients, the pathologist, and the chemist not engaged in cancer investigations, will profit from this book. And it will be helpful for the conscience of people who, having no real connection with cancer research, are often forced to judge the importance of various "sensational" cancer discoveries of a "tremendous importance."

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Subtilin—An Antibacterial Product of Bacillus Subtilis Culturing Conditions and Properties

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Introduction

Certain strains of *Bacillus subtilis* have been known for many years to be antagonistic to a number of pathogenic and non-pathogenic bacteria and fungi (1, 2, 3, 4, 5)[1]. A strain of *B. subtilis* has been investigated in this Laboratory because a crystalline product, apparently a polypeptide similar chemically to tyrothricin, was obtained directly from one of the first cultures when it was fractionated by the same procedure as used to obtain tyrothricin from *Bacillus brevis*. Although it appeared, at first, that *B. subtilis* and *B. brevis* produce similar antibacterial substances, as suggested by Dubos and Hotchkiss (12), additional work by the

* One of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[1] Rosenthal (6, 7, 8, 9, 10) found that several tyrothrix cultures, particularly *T. scaber*, possessed lytic activity against a number of organisms, living or heat killed, and that the filtrate of *T. scaber* was also active. The tyrothrix cultures were later identified as strains of *B. subtilis*. Recently he (11) tested a number of sporogenic bacilli of the subtilis-mesentericus group against living and dead Gram positive and Gram-negative bacteria. He found that 18 out of 31 strains were active against Gram-positive and Gram-negative organisms, and that all strains possessed lytic activity against the dead Gram-negative test bacteria. Dubos and Hotchkiss (12) stated that the tyrothrix cultures appear less active than the *B. brevis* culture with which these authors worked. They suggested, however, that the antagonistic action of the tyrothrix cultures was probably due to the alcohol-soluble, water-insoluble fraction produced by *B. brevis*, which fraction they named tyrothricin. Katznelson (13) studied the various members of the genus Bacillus for antagonistic activity toward fungi and bacteria. He found that *B. subtilis* produced a thermostable substance active against *Rhizoctonia solani*. It has also been reported that certain viruses are inactivated by *B. subtilis* cultures (14, 15).

present authors showed that the crystalline polypeptide from *B. subtilis* can be obtained free of antibacterial activity. Further, the antibacterial substance produced by *B. subtilis* has been found to possess certain properties that distinguish it markedly from tyrothricin. Its relative antibacterial activities are different from those of tyrothricin; it diffuses much more rapidly; it is less stable in the alkaline pH region; and in the crude state it is sensitive to light. The substance thus differing from tyrothricin has been named *subtilin* by the authors. Subtilin is then another type of antibacterial agent produced by a rapidly growing bacillus.

An investigation of the factors that affect the production of subtilin on synthetic media was undertaken so that production might be adapted to media prepared from various vegetable wastes or surplus agricultural materials. However, this report concerns only the results obtained with synthetic media. The use of synthetic media has facilitated the determination of some of the properties of subtilin by making it possible to work with solutions containing a minimum of unessential organic materials. The production of antibacterial substances by two species of the bacillus group grown on asparagus juice media has been reported previously from this Laboratory (16).

Experimental. Methods

Basic Medium. This was a mineral-salts-sucrose solution to which the constituents under study were added as desired. The composition of the solution was as follows[2]: $MgSO_4$, 0.5 g; KCl, 0.5 g; K_2HPO_4, 1.0 g; sucrose, 30.0 g; distilled H_2O, 1,000 ml. The media were adjusted to pH 7.1, sterilized at 10 lbs. steam pressure for 20 minutes, and incubated at 35°C. for 48 hours to confirm sterility. The pH after sterilization was 7.0.

Containers. Before sterilization, the media were dispensed in one or more of the following ways, depending upon the requirements of the experiments: 10 ml. quantities placed in 18 × 150 mm. test tubes; 50 ml. quantities placed in 250 ml. Erlenmeyer flasks; and 500 ml. quantities placed in Fernbach flasks.

Culture. Stock cultures of *B. subtilis*[3] were maintained on beef extract peptone agar.

The inoculum was prepared as follows: Transplants were made to fresh beef extract peptone agar slants; they were incubated at 35°C. for 24 hours; the growth was suspended in sterile distilled water; and the cells were washed three times by centrifuging and resuspending in sterile distilled water. Then 0.2 ml. of the final

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[2] All chemicals used in the basic solution were of reagent quality.

[3] The culture was obtained from Dr. N. R. Smith, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Dept. of Agriculture, who designated the culture as No. 231.

suspension was transferred to a tube containing 10 ml. of the desired medium, and incubated at 35°C. Transplants were made in three successive 24-hour periods, the final transplant being used to inoculate flasks of similar medium. A 4 per cent inoculum was employed throughout, that is, a 2 ml. inoculum was used for 50 ml. of medium and a 20 ml. inoculum for 500 ml. of medium.

Incubation Temperature and Time. All cultures of *B. subtilis* were incubated at 35°C. in the dark. Cultures in Erlenmeyer flasks were tested on the 3rd and 6th days. Cultures in Fernbach flasks were tested on the 4th day.

Tests. At the sampling periods, the following determinations were made on pooled triplicate flasks: The pH was determined by Beckmann pH meter; the residual sugar was determined by the Shaffer-Hartmann method; and the cell volume was obtained by centrifuging 10 ml. of macerated culture at 3,500 R.P.M. for 40 minutes. The cell volume and the amount of sugar utilized permitted only a rough estimate of the amount of growth.

Early in this investigation it was noted that cultures which possessed high activity gave an intense blue color, and those with little activity gave no color, on addition of $FeCl_3$. This test was run routinely in nutrient studies and was carried out by adding a few drops of a 1 per cent alcoholic solution of $FeCl_3$ to 5 ml. of culture. The test is characteristic of substances which are capable of forming a chelate compound with ferric ion; *e.g.*, catechol, salicylic acid, etc.

Test Organisms and Test Media Employed. Staphylococcus aureus (FDA No. 209) and *Micrococcus conglomeratus* (Merck "M.Y.") were cultured in nutrient broth[4] and incubated at 35°C.; *Lactobacillus casei* (ATCC 7469) was cultured in yeast-extract-glucose broth[5] and incubated at 30°C. All test organisms were subcultured by daily transfers in test media for at least 7 days prior to use.

Antibacterial Tests. The antibacterial tests were made on 3 or more replicate pooled cultures acidified to pH 2.5 and sterilized by autoclaving for 10 minutes at 10 pounds of steam pressure previous to testing. A 2-fold serial dilution of each culture was made in suitable liquid media. One loopful of a 24-hour broth culture of test organism was added to 5 ml. of each dilution. The tubes were incubated at the desired temperature for 24 hours, and then observed for growth of the test organism by comparison with controls. Observations were recorded as "no growth," "partial growth" (1+ and 2+), or "full growth" (3+). The antibacterial activities are reported in terms of concentration that gave approximately one-third inhibition of growth of the test organism after 24 hours of incubation; *e.g.*, if one-third inhibition occurred at a dilution of 1 to 1000 the activity was recorded as 1000. The one-third inhibition values were obtained by interpolation when not actually observed. A concentration of twice the level giving one-third inhibition was usually, although not always, completely inhibitory. The one-third inhibition

[4] The nutrient broth was composed of 3 g. of Difco beef extract, 5 g. of Difco peptone, and 1000 ml. of distilled H_2O; pH was 7.0; the broth was sterilized at 10 lbs. steam pressure for 30 minutes.

[5] The yeast-extract-glucose medium was composed of 10 g. of Difco yeast extract, 10 g. of C.P. glucose, and 1,000 ml. of distilled H_2O. It was sterilized at 10 pounds steam pressure for 20 minutes; pH was 6.8.

value was chosen because it varied less in replicate experiments than the value representing the dilution which gave no growth.

The reproducibility of the antibacterial test procedure used in this Laboratory was determined by testing a sample of tyrothricin against the three organisms in replicate on a single day and singly at intervals of two weeks over a period of twelve months. The greatest variations in activity observed in 20 replicates run on a single day were as follows: *M. conglomeratus*, 2-fold; *L. casei*, none; and *S. aureus*, 2-fold. Over a period of twelve months more than 90 per cent of the assays fell within the following maximum variations, respectively, for the three test organisms: 2-, 4-, and 8-fold. In comparison simultaneous antibacterial assays of 15 replicates of subtilin showed variations of 3-, 2-, and 4-fold, respectively, for the three test organisms.

Production of Subtilin

The microbiological production of antibacterial substances has been reported to be affected by trace elements, nitrogen source, and growth factors. Therefore these factors have been studied in relation to subtilin production. Although they generally affect growth as well as antibacterial activity, the chief criterion of a satisfactory medium in this work was production of antibacterial activity.

Effect of Trace Elements. The effect of trace elements on the production of antibacterial activity by various microorganisms has been noted by several workers. Waksman and Woodruff (18) stated that Fe seemed essential to streptothricin production. Kocholaty (19) found that substitution of Zn for Fe diminished the antibacterial activity produced by *Penicillium notatum* but increased growth; substitution of Cu gave high activity but poor growth; and substitution of Mn increased both the activity and growth. Bailey and Cavallito (20) found that addition of Fe to a synthetic medium containing inorganic salts, diammonium tartrate, and glucose resulted in an increase of crude citrinin.

It was found that lots of asparagine purchased from three different companies varied greatly in their ability to support the production of subtilin by *B. subtilis*, and further, that different lots of asparagine from the same company varied in this respect. This variation is illustrated in Table I. The increased antibacterial activity obtained by adding the ash from asparagine F, which gave high activity, to asparagine D, which gave the lowest activity, indicates a lack of essential inorganic nutrients[6] in asparagine D rather than presence of inhibiting agents. While all lots of asparagine tested supported growth, differences were noted in growth characteristics, pH course, and utilization of sugar.

[6] Richards and Troutman (17) have suggested that the trace elements present as impurities in asparagine may be an important reason for its growth-promoting value.

The results of experiments undertaken to determine trace-element requirements for subtilin formation with asparagine D as nitrogen source are reported in Table II. Trace quantities of Fe and/or Cu did not significantly increase the antibacterial titer over that obtained by their omission. However, Mn in the medium caused a considerable increase in antibacterial activity against all three test organisms. When Fe or Fe and Cu were added along with Mn still higher activities were observed

TABLE I

Variability in Subtilin Yields Using l(+)-Asparagine from Several Sources

The media consisted of 0.3% *l*(+)-asparagine from the various sources added to mineral salts-sucrose mixture

l(+)-Asparagine Source	Ash Content	Age	pH	Sugar utilized	*L. casei* activity*
	per cent	*days*		*per cent*	
D	0.03	3	6.40	4	80
		6	6.60	10	320
E	—	3	6.40	53	640
		6	6.30	76	20,000
F	0.33	3	5.45	63	>20,000
		6	5.80	73	>40,000
D+ Ash† from F	—	3	5.50	58	>80,000
		6	—	62	650,000

* The numbers represent the dilution of the culture at which one-third inhibition of the test organism occurred.

† A 3 g. sample of asparagine F was ignited at 550°C. overnight, the ash was dissolved in a few drops of conc. HCl, and the solution was added to 1 liter of medium made up using asparagine D.

in some cases. However, in replicate experiments, the results varied considerably particularly in the case of *L. casei* activity where a variation of as much as 100-fold was observed. Various combinations of Zn, B, Mo, and Ca added to the medium containing Fe, Mn, and Cu did not greatly alter the variations in activity.

The effect of added trace elements on subtilin formation when casein and tryptone media were used is shown in Table III; the data indicate that the casein and tryptone were deficient in trace-elements. In contrast with the results on asparagine medium, the trace elements did not appear to affect the growth markedly in these cases.

Effect of Nitrogen Source and Growth Factors. The specific effect of the nitrogen source in relation to antibacterial activity was investigated by

TABLE II

Effect of Trace Elements on Subtilin Formation

Basic Medium: Sucrose, mineral salts, and 0.3% $l(+)$-asparagine D

Trace Elements Added*	Age	pH	Cell Volume	Sugar Utilized	$FeCl_3$ Reaction	Activity *M. conglomeratus*	Activity *L. casei*	Activity *S. aureus*
	days		*ml./10 ml.*	*per cent*				
0	3	6.8	0.06	6	0		80	
	6	6.9	0.05	11	0		320	
Fe (1)	3	6.5	0.05	23	—	0	40	0
	6	6.7	0.05	23	0	0	320	0
Mn	3	5.5	0.18	30	—	200	5,000	50
	6	6.1	0.15	43	4+	500	>32,000	160
Cu	3	6.4	0.03	23	—	0	40	0
	6	6.7	0.03	25	0	0	160	0
Fe, Mn	3	5.6	0.53	29	4+	1,280	16,000	320
	6	5.3	0.50	54	—	5,000	12,000	640
Fe, Cu	3	6.5	0.03	4	0	20	10	0
	6	6.5	0.03	4	0	30	80	0
Fe, Mn, Cu (2)	3	5.3–5.6	0.4–0.8	20–40	4+	1,000–10,000	8,000–1,000,000	80–640
	6	5.3–6.0	0.4–0.8	50–70	4+	2,000–20,000	10,000–2,000,000	120–640
Asparagine F 0.3%	3	5.5	0.45	46	3+	>20,000	>650,000	1,280
	6	6.0	0.45	67	4+	>40,000	—	320

* Concentration (p.p.m.) of trace elements: Cu, 0.04 as $CuSO_4$; Fe, 0.03 as $FeSO_4$; Mn, 1.0 as $MnSO_4$.

(1) Concentrations of 0.01, 0.1, and 0.5 p.p.m. of Fe gave the same result as 0.03 p.p.m.

(2) These values represent the extremes of six separate experiments.

the addition of the desired nitrogen compound to a basic medium containing Fe, Mn, and Cu. Ammonium salts and urea supported negligible

growth, whereas glutamic acid and aspartic acid gave high but variable results comparable to those obtained with asparagine. The addition of organic growth factors[7] to asparagine and aspartic acid media had little effect on either the yield or reproducibility of yield of subtilin.

Dependency of Subtilin Formation on Strain. Another strain, *B. subtilis scaber*,[8] was tested for antibacterial substances. Under conditions of testing and culturing that would give high activity with the strain used in this work, namely, on a sucrose, mineral salt, and asparagine F medium, no activity against any of the test organisms was obtained although the amount of growth was comparable.

TABLE III

Effect of Addition of Trace Elements on the Production of Subtilin on Tryptone and Casein

Basic medium: sucrose and mineral salts

Nitrogen Source	Age	pH	Cell Volume	Sugar Utilized	$FeCl_3$ Reaction	Activity: *M. conglomeratus*	Activity: *L. casei*	Activity: *S. aureus*
	days		*ml./10 ml.*	*per cent*				
0.3% Casein*	3	5.1	—	23	—	0	40	0
	6	5.0	—	31	—	0	80	0
Casein + Fe, Mn, Cu	3	5.6	0.20	17	1+	640	4000	100
	6	5.5	0.48	18	2+	2560	6000	100
0.3% Tryptone	3	5.7	—	—	—	0	40	0
	6	5.4	—	—	—	0	80	0
Tryptone + Fe, Mn, Cu	3	6.2	0.93	63	1+	640	3000	200
	6	6.6	1.70	82	2+	1280	6000	200

* Merck and Co. according to Hammarsten.

Properties of Subtilin

Lack of Antibacterial Activity of the Crystalline Polypeptide and Culture Pigments. An inactive crystalline product was obtained, by fractionation, from an active *B. subtilis* culture grown on asparagus juice, and also from

[7] The growth factors were added to give a concentration in γ per liter of media as follows: Thiamin, HCl, 500; pyridoxin, 500; nicotinic acid, 250; pantothenic acid, 100; riboflavin, 200; *p*-aminobenzoic acid, 10; biotin, 1.0.

[8] Obtained from René J. Dubos.

an inactive culture grown on asparagine, the fractionating procedure being the same as that used for the isolation of tyrothricin from *B. brevis.* When 3 to 5 volumes of 1 per cent NaCl solution were added to an alcohol extract of *B. subtilis,* a substance slowly crystallized out of solution. The crystalline substance was apparently a polypeptide and, after five recrystallizations from methanol, had the following composition: C, 54.5 per cent; H, 7.7 per cent; N, 14.5 per cent; and substances reducing the Folin reagent as tyrosine 12 per cent. After acid hydrolysis approximately 60 per cent of the N reacted with HNO_2, and no glutamic acid could be detected. Unlike tyrothricin the purified peptide had no toxic effects when injected intraperitoneally into rats.

Because it was noted that cultures with high activity always had a yellow color, whereas other cultures, particularly those containing Fe but no Mn, had a pink color, tests of the pigments for antibacterial activity were made. Furthermore the pigmentation produced by the *B. subtilis scaler* strain, for which no antibacterial activity was detected on the medium used, was pink rather than yellow. The pink pigment was isolated by extracting the culture at pH 2.5 with isobutyl alcohol. The pigment was then removed from the isobutyl alcohol with dilute phosphate buffer at pH 7.8. When the phosphate buffer was acidified the pigment became soluble in ether. The pigment had absorption maxima at 400, 535, 561, and 614 mμ; showed an acid number of 12; reacted with benzidine in the presence of H_2O_2; and gave a stable pink color in concentrated H_2SO_4. On the basis of these properties, it was judged to be a metal porphyrin. The same pigment was isolated from the highly active Mn-sufficient culture but, in addition, a neutral yellow pigment was also obtained. This yellow pigment had weak absorption maxima at 355, 362, 393, and 425 mμ but was characterized no further. Neither pigment, at least on isolation, had any antibacterial activity.

Preparation and Antibacterial Activity of a Crude Dry Sample of Subtilin. The antibacterial substance in a culture of *B. subtilis* was stabilized in the following manner: Four-day cultures of *B. subtilis,* grown on asparagine media, dispensed in Fernbach flasks in 500 ml. quantities, were pooled and adjusted to pH 4.7 by the addition of concentrated HCl. After two hours at room temperature the material was centrifuged in a Sharples Super Centrifuge. The precipitate, which included cellular debris and intact cells, was dried to a thin film in a vacuum desiccator over P_2O_5. The dried material was then ground in a Wiley mill. The yield of this crude material, a pink powder, was 1.8 g./1. Determination of its

activity by suspension in acid solution at pH 2.7, sterilization, and assay, showed that most of the activity of the original culture was retained.

Using the test conditions described under "Methods," only 0.5γ per ml. of this *crude* material was necessary to inhibit the growth of *M. conglomeratus*, whereas 0.2–0.5γ per ml. of *isolated* tyrothricin was necessary to cause the same inhibition. The U. S. N. Medical Research Unit No. 1[9] tested crude subtilin and found that it was at least as effective as isolated tyrothricin in inhibiting the growth of *S. aureus* and *Streptococcus viridans*. They also found that subtilin had a bactericidal action on the organisms tested. High concentrations of subtilin failed to inhibit the growth of *Eberthella typhi*.

Separation of the Active Substance from the Dry Powder. The active substance was removed from the dried powder by warm ethanol extraction. When 0.2 g. was extracted with 5 ml. of 95 per cent ethanol for one-half hour, little of the active material went into solution. However, when 0.2 g. of powder was first wet with 0.5 ml. of water and then extracted with 4.5 ml. of warm ethanol, most of the activity was found in solution. A study of solubility of the active substance in dilute ethanol was not made. When the powder was treated with warm methanol instead of ethanol, inactivation took place. Similarly, the activity was lost when the powder was treated with ethanol containing trace quantities (0.04 per cent) of formaldehyde.

The active material in freshly prepared ethanol solutions was precipitated slowly by the addition of two volumes of 1 per cent NaCl solution, and more rapidly by adding two volumes of H_2O and adjusting to pH 2.1. The precipitate obtained by the latter method inhibited *M. conglomeratus* in concentrations varying from 0.06 to 1.0γ per ml., depending on the time of storage of the powder prior to extraction. This precipitate contained 5.7 per cent nitrogen and gave a blue color with alcoholic solution of $FeCl_3$. A test of the unhydrolyzed material with the Folin reagent for phenols showed that it contained 33 per cent of a phenolic substance calculated as tyrosine. If the phenolic material were tyrosine, it would be present in a ratio of one tyrosine to two N atoms. No —SH group could be detected in the acid precipitate.

Compared to tyrothricin, the molecular weight of subtilin appears to be low. This was judged by a comparison of the diffusion rates of subtilin, tyrothricin, and penicillin in 1.5 per cent agar solution. For example,

[9] Private communication from Captain Albert P. Krueger.

using the agar-cup-plate method of assay, tyrothricin diffuses little, if any, into the surrounding medium, while subtilin diffuses at a rate comparable to that of penicillin.

Stability. When the active material, dried in a vacuum desiccator as described above, was stored for six months at 5°C., most of the activity toward all three test organisms was lost. This may have been due to oxidation by molecular oxygen. Alcohol solutions of subtilin were unstable when stored at 5°C., losing all of the original activity against *L. casei* after 15 days' storage at this temperature.

When whole cultures, or suspensions of the dried powdered material, were steam sterilized for 10 minutes under 10 pounds of pressure, activity was destroyed, provided the pH was greater than 7.0. The activity was least affected at pH 2.5. Hence in all of the nutrient studies, the whole culture was sterilized at this acid pH for purposes of assay.

The effect of several storage conditions on the activity of the whole culture was studied. One sample of a high-activity culture was adjusted to pH 5.4, and another sample of the same culture was adjusted to pH 2.7. Unsterilized samples from each lot were stored at 0°C. Sterilized samples from each lot were held in the dark at room temperature, at 5°C., and at 0°C. One sample was held at room temperature and exposed to diffused sunlight. All samples were kept in Erlenmeyer flasks, covered with Parafilm paper, and were tested at 7, 9, and 12, or 14 days for antibacterial activity. Heat-sterilized cultures of *B. subtilis* were found to be light sensitive. No loss of activity was noted in cultures stored 12 days in the dark at room temperature, but samples stored in the light at the same temperatures retained only very little of their original activity at either pH. Storage in the dark at 5°C. and 0°C. for 14 days had no apparent effect on the activity of sterilized or unsterilized cultures. Although the effect of light on the production and stability of subtilin during culturing was not studied, it is likely that more consistent results would be obtained by culturing in the dark. The observed effect of light on subtilin recalls the observation by Reid (21) and others on the effect of light on penicillin.

Discussion

The variation of subtilin formation on a given medium was considerable, particularly as measured by *L. casei*-inhibiting activity, where a variation of as much as 100-fold was observed. Since such variations are well beyond the limits of error of the test methods, the explanation may lie in variation in production of antibacterial substance. This explanation

would necessarily include the formation of at least two active agents differing in their relative activity on the test organisms. It is believed that this variation is due to some uncontrolled factor or to a marginal factor, such as trace element concentration or temperature of incubation, which might cause marked differences in yield of antibactrial product as a result of small variations.

A $FeCl_3$-reacting substance possessing no activity has been obtained from active cultures. However, during purification procedures, activity was never obtained unless the $FeCl_3$ reaction was positive. Proof that this material is in whole or in part an integral part of the subtilin molecule must await ultimate purification. Lemoigne (22) has shown that *B. subtilis* and other soil organisms produce a $FeCl_3$-reacting substance during the early stages of their growth on a sugar-mineral-salt medium. He refers to this substance as an *o*-diphenol. Recently Abraham, *et al.* (23) have isolated inactive penicillamine as a characteristic hydrolysis product of penicillin. Penicillamine, $C_6H_{11}O_4N$, reacts with ninhydrin to give an intense bluish-purple coloration, and with $FeCl_3$ to give a deep blue coloration.

The antibacterial substance produced by *B. subtilis* has little in common with tyrothricin. The relative activity toward test organisms is different for the two substances; *i.e.*, on the basis of equal *M. conglomeratus*-inhibiting activities, subtilin is about four times more effective against *S. aureus* and about 100 times more effective against *L. casei* than is tyrothricin. Subtilin is extremely sensitive to heat at pH 8, whereas tyrothricin is reasonably stable at that pH. The instability of subtilin during storage in alcohol solution or in the dry state is far removed from the stability of tyrothricin, or gramicidin. On the other hand the properties of subtilin suggest that it may be similar to penicillin.

Summary

Cultures of high antibacterial activity were produced by growing *B. subtilis* on synthetic media consisting of mineral salts, sucrose, asparagine, and trace amounts of Fe, Mn, and Cu. Under the conditions used, Mn was the only element that increased the activity when employed alone. The addition of organic growth factors had little effect on the activity production. Aspartic and glutamic acids, but not urea or inorganic nitrogen, were also suitable nitrogen sources. Tryptone and casein without added trace elements supported good growth, but very little antibacterial activity was obtained unless trace elements were added. A

substance giving a strong blue color with $FeCl_3$ was always found in cultures possessing high activity. The antibacterial activity obtained with a given medium was quite variable. This variation is thought to be due to some uncontrolled marginal factor.

The antibacterial substance is active against *S. aureus*, *L. casei*, *M. conglomeratus*, and *S. viridans;* but not against *E. typhi.*

The product has been extracted from the dried organisms with warm ethanol. Sterilized cultures of *B. subtilis* retain their antibacterial activity for 12 days when standing in the dark at room temperature, but lose 90 per cent of their activity in the light. The active substance is fairly heat stable. It appears to be most stable in the acid region near pH 2.5, being unstable above pH 7. Its stability properties and rapid rate of diffusion distinguish it from tyrothricin. The active product of *B.' substilis* has been called *subtilin* for convenience, although the activity is probably due to more than one substance.

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The Biochemistry of *Vibrio cholerae*

III. Acid Regulation by Means of the Carbon-Dioxide-Bicarbonate Buffering System

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Introduction

Studies of the growth of *Vibrio cholerae* in a simple liquid medium (1, 2) indicated that glucose was a factor regulating the extent of reproduction. Greater glucose utilization always resulted in a greater crop of vibrios. The use of glucose by the vibrios was limited in this medium, however, by accumulation of acid. Digestion of glucose amounting to about 0.3% of the weight of the medium lowered the pH to a point at which the organisms ceased to multiply. Retarding acidification might therefore extend the period of multiplication.

Hirsch (3) reported increased growth in the presence of $CaCO_3$. Under the conditions of our experiments, the addition of $CaCO_3$ did not result in greater yields of vibrios. Periodic addition of alkali also gave disappointing results. The vibrios are highly sensitive even to a transient exposure to high alkalinity, and injury may well have occurred to a portion of the population before the added alkali could be dispersed through the medium.

The best means of retarding acidification would seem to be an efficient buffer system. Such a system should operate in the region near neutrality (2). It should consist of materials which are not harmful to the organisms in the concentrations needed. Materials which might actually be useful *per se* would of course be preferable.

The fact that our cultures were being subjected to vigorous aeration suggested the possibility of establishing a carbon dioxide exchange system similar to that in the lung. A considerable reserve of alkali in the form

of sodium bicarbonate can be maintained at a desired pH by proper adjustment of the carbon dioxide content of the atmosphere. Since the atmosphere in the culture flask is constantly replaced, carbon dioxide released from this bicarbonate reserve by the formation of acid is immediately swept away.

The use of this system has led to the production of extremely dense populations of *Vibrio cholerae* in a water-clear medium which does not interfere with the use of the culture as a direct vaccine.

Methods

Measurement of pH and Turbidity. The difficulties involved in removing samples of the culture for external examination were circumvented by determining pH and turbidity within the vessel itself. There could be no loss of carbon dioxide before the pH was measured nor contamination of the culture from frequent openings of the flask. It was unnecessary to interrupt the flow of gases or to continually diminish the total volume of the culture.

Electrodes designed for use in electrometric titrations were mounted in a glass sleeve which permitted their insertion directly into the medium. The sleeve was mounted in a rubber stopper which fitted the neck of the flask. The assembly was sterilized by exposure to formalin fumes between periods of use and rinsed several times with sterile distilled water before placing in a new culture.

The sampling tube described by Shwartzman and Bierman (4) was modified to permit the culture to be forced up into the tube at will and held there while turbidimetric measurements were made. The column of liquid was then permitted to drop back into the flask. The sampling tube was mounted in one of the side necks when a three-necked flask was used as a culture vessel.

Control of Aeration. The concentration of carbon dioxide was regulated by measuring the rates at which CO_2 from a commercial cylinder and air from the laboratory compressed air supply flowed into a common delivery tube. Simple, easily constructed flowmeters of the Venturi U and capillary type were found quite satisfactory.

Fluctuations in the flow of compressed air were largely eliminated by the T-type of escape mechanism. This consisted of a long small-bore side arm from the air-line. The T-tube opened at the bottom of a three-foot column of water, permitting the escape of air when the pressure reached the desired amount.

Aeration was carried out by means of the cloth bubblers previously described (1). The great mass of tiny bubbles produced by this device has a great advantage in maintaining the CO_2 equilibrium between the atmosphere and solution even during the most rapid acidification of the medium.

Cultural Technic

Preparations. The following items were sterilized by autoclaving:

1. The culture vessel, a three-liter round-bottomed Pyrex flask, which may be three-necked, with the bubbling and sampling tubes mounted in rubber stoppers

in the small necks, or an ordinary flask with these attachments permanently sealed through the walls. At the time of autoclaving it contained 1 l. of distilled water and 15 ml. of casein digest (1), and was stoppered with cotton.

2. The desired amount of glucose (for routine work, 10 g.) dissolved in a minimum amount of water.

3. Twelve g. of $NaHCO_3$, wrapped in heavy paper and placed in a Petri dish. When cool, the glucose and bicarbonate were added to the culture vessel and the mixture shaken until the bicarbonate had dissolved. Even when sterilized as directed, there was a variable amount of carbonate formation from the bicarbonate, so that it was advisable to bubble carbon dioxide through the completed medium for a few minutes before inoculation.

Inoculation. A culture of the vibrio—in our work, strain V.c. 35—was grown in 100 ml. of salts-C-D medium (1) without aeration. The entire culture was used to inoculate the new medium. Best results followed the use of an inoculum which was just beginning to show an observable increase in turbidity. Older inocula may cause abnormally long lag periods. If the projected study included pH determinations, the cotton stopper in the culture vessel was replaced at this time by the rubber stopper bearing the electrodes.

Incubation. The culture was incubated at 37° C. until no further increase in turbidity was observable, a period usually of about 24 hours. It was not possible to follow the progress of the culture by observing the pH variations as in previous cultures, since the pH might continue to drop after growth had apparently ceased if sugar was still present, or might rise if it was not. Aeration with a controlled mixture of carbon dioxide and air was continued throughout the incubation period.

Aeration. The percentage of carbon dioxide was arbitrarily adjusted to any value between 10% and 30% by means of the flowmeters. If preferred, the mixture may be adjusted to produce and maintain a pH a little above 7 without reference to the flowmeters, though it is then desirable to note the rates of flow thus established in order to distinguish later between pH changes due to acidification and those due to possible variations in flow of CO_2.

Turbidity Measurements. The development of the culture was followed by comparing suitable standards with the column of culture, which was raised into the sampling tube. The standards were the silica suspensions used in previous work (1, 2), in containers conforming to the dimensions of the sampling tube. In order to make exact measurements of turbidity at the end of the experiment, diluted samples of the culture in the standard-sized tubes must be used, since the subjective error in measuring the turbidity of heavy suspensions in thin layers is probably considerable.

Characteristics of the BRF Culture

The new type of culture differs markedly from previous preparations (2), which for convenience will be referred to as salts-C-D cultures, in regard to glucose utilization, pH changes during growth, and total crop. In the account of these differences which follows, the new product is spoken of as the BRF (Biochemical Research Foundation) direct vaccine, and the culture itself and the method by which it is produced similarly designated.

Glucose Utilization. In contrast to salts-C-D cultures which were unable to digest more than 3 g. of glucose per liter, the new BRF cultures completely utilized all of the glucose when as much as 1% was made available. Less than this concentration resulted in smaller final crops. When greater amounts were offered, more than 10 g. per liter might be assimilated, but some glucose would still remain in the medium. When 20 g. of glucose were incorporated in the medium about 7 g. still remained after multiplication had ceased. Of an original 50 g. about half was used up. Such "forced feeding" of glucose, however, did not appear to increase the growth appreciably over the crop obtained when 10 g. of glucose were originally present and completely utilized.

The amount of acid formed in the older type of culture was roughly estimated by titration of uninoculated medium to the end pH of 5.5 with sulfuric acid. The results indicated that in the Salts-C-D medium without added CO_2 each mole of glucose gave rise to approximately two moles of acid. Under the conditions pertaining to the BRF cultures such a titration is even less accurate due to the powerful buffering system. The results in this case were tentatively interpreted to indicate the formation of about three moles of acid for each mole of glucose destroyed.

The pH Changes During Growth. The initial pH of the medium containing bicarbonate is largely dependent on the partial pressure of CO_2 in the atmosphere. In an unaerated culture, formation of acid from the glucose would release CO_2 from the bicarbonate, increasing the partial pressure and lowering the pH. A sufficiently rapid replacement of the atmosphere combined with a maximum liquid-gas interface area as provided by the cloth bubbler sweeps this gas away as quickly as it is formed, however, and the pH remains essentially constant.

The partial saturation of the medium with carbon dioxide before inoculation lowers the pH to a point near the desired value so that equilibrium between the medium and the aeration mixture is quickly established. As anticipated from previous work, a pH near neutrality proved most desirable. The amount of CO_2 needed to establish this pH varies, of course, with the amount of bicarbonate. Good growth could be obtained with any concentration of CO_2 from 5% to 95% provided the appropriate amount of bicarbonate was employed. Too little bicarbonate, however, permitted acidification of the culture, but 12 g. per liter gave excellent results which were not improved upon by the use of greater amounts. With this quantity of bicarbonate any concentration of CO_2 between 5% and 30% was satisfactory. The range of pH represented by these concentrations is roughly 7 to 8.

The contrast between the extreme changes of a salts-C-D culture and the stability of a BRF culture in respect to pH are illustrated in Fig. 1. The BRF culture chosen for this representation was one in which an

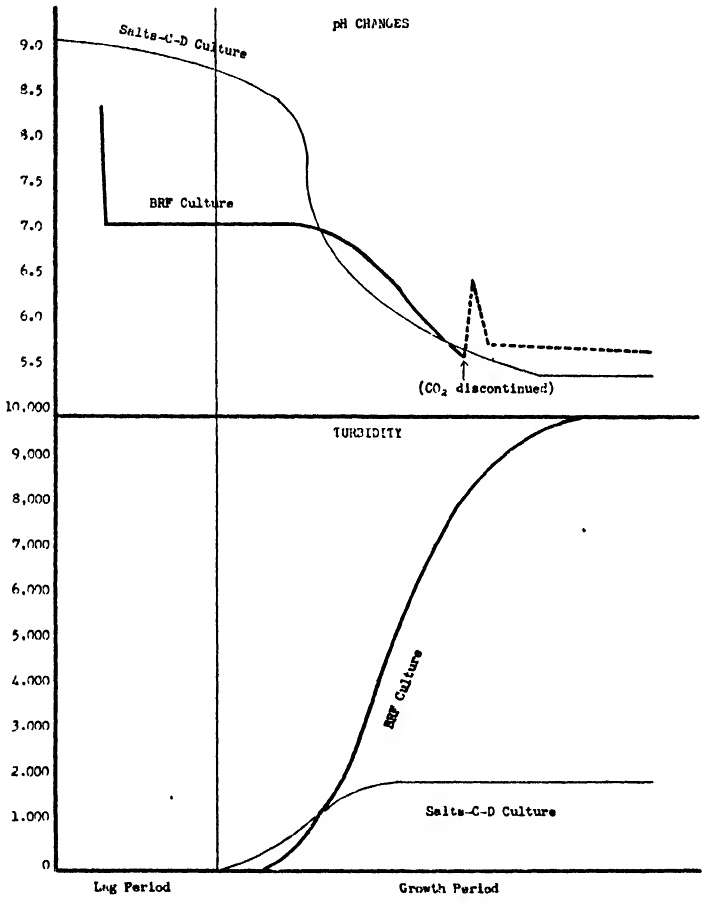

Fig. 1

Comparison between Variations in pH and in Turbidity during Growth of Salts-C-D Cultures and of BRF Cultures

unusually great depression of pH was encountered, while the salts-C-D graph represents an average experiment. The time abscissae of the BRF graph have been foreshortened and the origin displaced in order to permit direct comparison of the growth periods.

The initial steep drop in the pH of the BRF culture is due to the ten minute period during which equilibrium with the aerating gases was being established. This particular experiment was one in which an abnormally long time elapsed before the growth period was initiated. However, this portion of the curve serves to illustrate the accuracy with which the pH is maintained. It will be noted that the culture had already progressed further than the final turbidity of the salts-C-D culture before the pH began to decline from this level.

The BRF cultures rarely varied more than 0.3 pH units in the full course of development, when 12 g. of bicarbonate and 20% CO_2 were employed. Attempts to maintain the pH within still narrower limits by diminishing the CO_2 at the first sign of acidification did not lead to any great improvement of the final crop. Longsworth and MacInnes (5) found that strict maintenance of the pH of *L. acidophilus* greatly enhanced growth, but the improvement in this case was in comparison with the growth obtained by the more usual technic of leaving the regulation of acidity to chance buffers in the medium. As compared with usual cultures, the BRF culture may be said to be maintained at a constant pH.

As previously stated, the culture chosen for illustration represents an extreme degree of acidification. Since the lethal pH of 5.5 appeared imminent the CO_2 was discontinued near the end of the experiment. The subsequent changes in pH are indicated on the graph by means of a dotted line. The sharp rise is attributable, of course, to the immediate loss in dissolved CO_2. The subsequent equally sharp drop appears too extreme to be accounted for on the basis of increased population. It seems more likely that the higher pH is more favorable for the activity of enzymes already present in the medium, so that the rise in pH results in more rapid sugar degradation and consequent acid formation. As the pH again approaches the value at which the CO_2 was discontinued the rate of acidification slows down until it becomes the same as would have been found if the interruption had not occurred. It is possible that 5.5 is the terminating pH for a cholera culture because certain glucose-splitting enzymes become inactive at this point.

Nitrogen Utilization. It was found that very good growth could be obtained occasionally when no nitrogeneous matter other than that supplied by the inoculum was incorporated in the medium. The irregularity of results, however, prompted us to include the additional casein digest as a routine procedure, since the material could be completely removed by dialysis when desired.

The Finished Product. Sodium silicate standards contained in half-inch test tubes were used in previous work to determine the extent of growth. Two thousand parts per million of fuller's earth, prepared according to the specification of the American Public Health Association, is about the most concentrated standard that can be used by this method. With the salts-C-D medium, this turbidity was rarely exceeded. A culture equivalent to 1,500 p.p.m. was considered very good.

With the BRF method, however, cultures were much too turbid to be compared directly with such standards. In order to evaluate them it was necessary to dilute the cultures 1/10. In such dilutions the final crops usually had a turbidity equal to 750 or 1000 p.p.m. of silica, implying an actual turbidity equal to that of 7,500 to 10,000 p.p.m. if it were possible to use such standards. The new method, therefore, gave rise to a five-fold increase in the yield of cholera vibrios.

A graphic representation of this difference is presented in Fig. 1. It will readily be seen that growth reaches a much greater total value. In its final state the culture, which when seeded was indistinguishable from clear water, is very nearly opaque. The growth period tends to last longer and ends less abruptly in BRF medium than in the older medium. The terminal pH is usually considerably above that of the extreme shown in the graph. An alkaline culture is not unusual.

The addition of 0.25 g. of phenylmercuric acetate dissolved in a small amount of hot water effectively sterilizes the culture in less than 5 minutes, without interfering with its use as a vaccine. The efficacy of this preparation as an immunizing agent has not been fully determined as yet, but it has been injected subcutaneously in volunteers with no reaction of an objectionable nature.

The preparation may be concentrated under vacuum to a small volume, then dialyzed to remove residual substrate materials. Drying by pervaporation leaves a pure mass of vibrios and high molecular products of their growth which serves as an excellent source of subject matter for chemical and physical studies.

Discussion

While it is apparent that utilization of more glucose than was possible in previous cultures has resulted in greatly improved crops, further improvement along these lines is not to be expected since glucose digestion is no longer a limiting factor. More glucose may be used than is necessary to produce a maximum crop, and the digestion products no longer inter-

fere with the action of the enzyme. It now appears that approximately 1% is the upper limit of glucose concentrations which will result in greater growth, though glucose may continue to be digested.

The principal action of the bicarbonate-CO_2-mixture in the BRF culture is obviously that of a buffer. The familiar problem as to whether CO_2 plays some more direct role in the metabolism of the vibrio, however, remains unanswered. In common with most bacteria (6), *Vibrio cholerae* requires a certain amount of CO_2 to grow at all. This amount is very small and it seems unlikely that its value could be ascribed to buffering action alone, especially in an unaerated culture.

The BRF method owes it success to the intimate mixing of gases with the culture media, lending re-emphasis to the importance of aeration as a means of insuring maximum growth, as stressed by Rahn and Richardson (7).

Summary

The CO_2 exchange between $NaHCO_3$ in the medium and the aerating gases may be used to stabilize the pH of cultures of *Vibrio cholerae.* This buffering action permits growth of extraordinarily heavy cultures in a medium consisting only of glucose, bicarbonate, and a small amount of amino acids and salts, when aerated with CO_2 enriched air.

Such cultures may be killed by the addition of phenylmercuric acetate and used directly as a vaccine, or as a readily purified source of vibrios for chemical and physical studies.

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Physico-Chemical Studies on the Water-Soluble Fraction of Powdered Wool*

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Introduction

Several of the characteristic properties of wool keratin are altered by prolonged grinding in a steel ball mill (1, 2). The most outstanding alteration is in the solubility of the protein. An increasing fraction of the powdered wool becomes soluble in water as the grinding is prolonged. The dry grinding of other proteins has resulted in similar solubility changes as recently shown by Cohen (3).

Since the most extensive chemical alteration is in the soluble fraction, a more thorough study of this fraction promised to aid in an explanation of the changes that occur in the protein structure during the grinding process.

This paper presents chemical and physico-chemical studies of the water-soluble components of powdered wool.

Experimental

Powdered wool was prepared essentially as previously described (2); samples (100–200 g.) were ground by 300,000 to 1,500,000 revolutions of the ball mill, and material that passed a 100-mesh sieve was used for subsequent analyses. As the grinding proceeded, the color changed from light tan to gray. Iron found in the product was attributed to abrasion of the ball mill.

The powdered wool was suspended in water, stirred for about three hours, centrifuged, and the supernatant liquid was diluted to volume.

Total nitrogen of the extracts was determined by the Kjeldahl method; biuret nitrogen, by the method of Robinson and Hogden (4). A calibration curve for the

* The experimental data are taken from a dissertation submitted by T. Urban Marron to the Faculty of the Graduate College of the State University of Iowa in partial fulfilment of the requirements for the degree of Doctor of Philosophy, July, 1942.

latter in terms of total nitrogen was prepared from biuret nitrogen values of powdered wool ground for 300,000 revolutions and dissolved in 0.5 N NaOH; although soluble in dilute alkali, this powdered wool had not yet been appreciably altered chemically by the short period of grinding. The colorimetric values exhibited a straight line relationship to the total nitrogen values of the samples.

The pH of the extracts was determined with a glass electrode. Cystine was determined by the Shinohara method (5); tyrosine, by the Folin-Ciocalteu method (6).

Fractionation of the soluble material by electrophoresis was suggested by the work of Williams (7). Electrophoresis was carried out in a series of 5 to 6 cells in a long water bath maintained at 20°-30°C. Three sets of cells were constructed. The two larger sizes were made similar to those of Spies, *et al.* (8) from 500 and 125 ml. Erlenmeyer flasks. The "micro" cells of about 12 ml. capacity were constructed as horizontal cylinders with closed ends from 22 mm. pyrex tubing with top openings made to take a #1 rubber stopper. The side arms were of 7 mm. tubing entering just below the center of the cell ends. The electrode cells for the micro train were flat-bottom glass bottles made from 34 mm. pyrex tubing fitted with flat, heavy platinum foil electrodes (2 × 1.7 cm.)

Direct current up to 5000 volts was supplied by vacuum tube half-wave rectification of transformed alternating current. The current that passed through the solution seldom exceeded 10 milliamperes because of the low conductivity.

Iron salts in the extracts separated as solid hydrated iron oxide soon after the pH gradient was established. When the oxide did not clog the passage between cells it was left in the set until the end of the run, otherwise it was filtered off, and the solutions were returned to their respective cells for continuation of electrophoresis.

When the extract was placed in one of the large cells (500 ml.), and water in the others, it was found that the conditions (7) of suitable pH gradient, dilution, and low conductivity were fulfilled, and that migration produced varying concentrations of protein material in the different cells.

In subsequent experiments it was more advantageous to use the medium cells (125 ml.), each of which was filled with the extract at the beginning of a run. The fraction that accumulated in any one of these cells could be subjected to another period of electrophoresis in the micro cells.

For polarographic studies of the extracts a direct reading Fisher Electropode was used. The measured potential was supplied by an outside source of two dry cells in series, through a Leeds and Northrup Students' potentiometer and a dial type resistance box. Nitrogen was bubbled through the solution until oxygen was expelled, then over the solution while readings were being taken. Electrical connections were made through an external saturated calomel half-cell from which a saturated KCl-agar bridge dipped into the side arm of the electrolysis cell. Samples of the wool extracts were electrolyzed at the dropping mercury electrode after dilution of 1.0 ml. to 50 ml. with a cobalt buffer. Residual currents were plotted to −0.9 volt against the saturated calomel electrode, and were then assumed to continue in a straight line with the same slope (9). Wave heights were all measured between lines drawn parallel to the extrapolated residual current through midpoints in the plateaus (10). Wherever a maximum occurred the line was passed through the lowest point of the depression immediately following.

The State of the Ground Wool Extracts

It has already been shown that considerable oxidation of the cystine of wool occurs during grinding (2) and that there is a correlation between the oxidative changes and solubility. To determine whether the changes in solubility occurred during grinding or as a result of further oxidation in the extraction process, extracts were made while oxygen or nitrogen was bubbled through the mixture or after addition of hydrogen peroxide; the small increases in soluble and biuret nitrogen indicated that the only significant changes occurred in milling.

The increased solubility of powdered wool with continued grinding was accompanied by increased acidity. After 500,000, 750,000, and 1,000,000 revolutions[1], samples of 2.5 grams extracted with 100 cc. water yielded 8, 23, and 45 mg. of soluble nitrogen, respectively, with pH values of 5.9, 5.2, and 4.8.

Since addition of acids or of alcohol or acetone to the extracts produced no precipitation, it appeared probable that the molecular size of the soluble material was relatively small and that the amphoteric nature of the original protein had been altered.

Total and Biuret Nitrogen Relationships

As protein nitrogen is more accurately indicated by biuret than by total nitrogen, the relation between total and biuret nitrogen of the extracts was investigated. The extracts of grinding B, C, D contained respectively 84.5, 87.3, 85,7 per cent of their total nitrogen in the form of biuret nitrogen. Thus it appeared that the grinding produced increasing amounts of a soluble fraction of relatively homogeneous composition. However, during electrophoretic fractionation, non-protein nitrogen apparently moved out of the acidic cells into the cathode cell leaving behind material that more closely approached a 1:1 ratio of biuret N to total N (Table I). Control experiments showed that the total amount of biuret nitrogen in an extract did not change during electrophoresis.

Tyrosine and Nitrogen Relationships

To discover whether the various cells after electrophoresis contained chemically different protein decomposition products, the relation between tyrosine and total nitrogen was determined. The Folin-Ciocalteu method measures both tyrosine and tryptophan, but since the trypto-

[1] Hereafter, wool ground for 300,000, 500,000, 750,000, 1,000,000, 1,500,000 revolutions will be referred to as grinding A, B, C, D, E respectively.

phan content of wool is small (0.7%) compared to tyrosine (4.5%), the results are expressed as tyrosine. The tyrosine content of the soluble material in extracts of grinding A, B, C, D was 5.5, 6.0, 4.0, 4.3 per cent, respectively. These values indicate either a decrease in extractable tyrosine or an increase in the destruction of this amino acid as the grinding proceeded.

TABLE I

Nitrogen Content of Electrophoretic Fractions

Cell	pH after electrophoresis	Biuret N*	Total N*	$\frac{\text{Biuret N}}{\text{Total N}}$
1 (anode)	2.2	0.35	0.36	0.964
2	3.3	0.35	0.39	0.899
3	4.0	0.37	0.38	0.966
4	5.6	0.34	0.34	0.982
5 (cathode)	12.0	0.19	0.36	0.539
Original solution	4.8	0.33	0.39	0.838

* Biuret and total nitrogen expressed as mg. per ml.

A sample (10 g.) of grinding D was extracted (total volume, 550 ml.) and subjected to electrophoresis for 72 hours in the 125 ml. cells.

TABLE II

Tyrosine Content of Electrophoretic Fractions

Cell	pH after electrophoresis	Total N*	Tyrosine N*	$\frac{\text{Tyrosine N}}{\text{Total N}} \times 100$
1 (anode)	2.1	26.2	1.22	4.6
2	2.8	24.0	1.31	5.4
3	4.5	26.0	0.96	3.8
4	8.7	23.3	0.75	3.2
5 (cathode)	11.9	20.5	0.10	0.5

* Total and tyrosine nitrogen expressed as mg. per 100 ml.

A sample (10 g.) of grinding D was extracted (total volume, 500 ml.) and subjected to electrophoresis for 45 hours in the 125 ml. cells.

After electrophoresis of an extract of grinding D (Table II) the fraction with tyrosine content approaching that of native wool (5.2%) had a pH (2.8) well below the isoelectric point of wool. A similar disparity appeared when tyrosine nitrogen was related to biuret nitrogen. The calculated amount of tyrosine present in the original extract was accounted for after electrophoresis indicating that the amino acid had not been destroyed by this operation.

Successive Electrophoretic Fractionations

During electrophoresis of extracts in the 125 ml. cells, nearly all titratable acid and alkali moved into the electrode cells. To avoid this and to make the current more efficient in transporting the protein, some workers have replaced the electrode cell solutions with conductivity water (8). Or, when the cell train was long, the end cells containing the acid and alkaline material could be removed, with a shift of the electrodes one cell toward the center.

A third method was devised which had the advantage of spreading the material from one 125 ml. cell throughout five or six micro cells without diluting the extract. After a short period of electrophoresis

TABLE III

Re-electrophoresis of Electrophoretic Fractions

	Section A						Section B					
	Parent Solution 90 hours		Cell #2 of Parent 64 hours		Cell #3 of Parent 64 hours		Parent Solution 60 hours		Cell #2 of Parent 24 hours		Cell #3 of Parent 90 hours	
Cell	pH	N*	pH	N	pH	N	pH	N	pH	N	pH	N
1 (anode)	2.2	.35	2.4	.52	2.9	.71	2.0	.23	2.4	.36	2.9	.35
2.......	3.3	.35	2.9	.45	3.9	.55	3.0	.25	2.8	.39	3.6	.31
3..	4.0	.37	3.5	.33	4.4	.37	4.0	.26	3.2	.26	4.2	.34
4...	5.6	.34	4.1	.28	4.5	.20	9.3	.24	3.7	.25	4.5	.28
5**......									4.4	.22	5.3	.16
6 (cathode)...	12.0	.19	9.0	.11	9.9	.11	11.8	.17	9.7	.12	9.9	.14

* N = Biuret nitrogen expressed as mg. per ml.

** Six cells used only in last two runs.

the electrolytes had moved into the end cells; the protein fraction in any one of the center cells could be transferred to the set of micro cells and subjected to another period of electrophoresis.

Typical data on extracts of grinding D are summarized in Table III. Samples (10 g.) of the powder were extracted with 500 ml. of water and put into a set of five 125 ml. cells. After preliminary electrophoresis, the contents of cell #2 and later of cell #3 were removed and subjected to re-electrophoresis while the rest of the cells were stored in a refrigerator. Control experiments showed that four days refrigeration caused no change in pH, biuret values, precipitable material, or polarographic waves of extracts. Section B of the table contains the data of a similar run in which the time of electrophoresis was varied and the number of cells was increased from five to six.

Data in the table indicate that the electrophoretic fractionation of cells 2 and 3 separated some characteristically different materials. Williams (7) has already suggested that variation in electrophoretic fractionation of proteins with respect to acidity and peptid composition indicates chemical difference.

Molecular Size from Precipitation Data

Precipitation studies were used to estimate the size of the particles in the soluble portion of powdered wool and its electrophoretic fractions.

TABLE IV

Precipitability of Electrophoretic Fractions

	1 (anode)	2	3	4	5 (cathode)
Extract of grinding D, after 24 hour electrophoresis					
(A) pH	2.2	2.7	3.6	9.9	11.5
(B) N precipitated by tungstic acid	36.5	34.5	**	46.1	25.2
(C) N precipitated by trichloroacetic acid*	15.0	14.8	**	26.0	6.1
(D) Differential (B) − (C)	21.5	19.7	**	20.1	19.1
Extract of grinding E, after 90 hour electrophoresis					
(A) pH	1.8	2.5	3.6	7.8	11.6
(B) N precipitated by tungstic acid	5.5	12.3	31.3	41.0	25.7
(C) N precipitated by trichloroacetic acid	2.4	7.7	10.2	8.5	3.0
(D) Differential (B) − (C)	3.1	4.6	21.1	32.5	22.7

* Figures for N are expressed as per cent of total N of the cell contents.
** Cell contents used for re-electrophoresis.

Gelatin and Witte's peptone were used as reference material; the precipitating agents were 20 per cent trichloroacetic acid and a solution of tungstic acid and sodium sulfate (11). Biuret nitrogen determinations were made on the original solutions and on the filtrates.

Trichloroacetic acid removed 7.4 per cent of the biuret nitrogen from a peptone solution whose nitrogen content was approximately that of the wool extracts, whereas the tungstic acid reagent removed 51.8 per cent. From gelatin solutions of a similar concentration all of the peptid nitrogen was precipitated by tungstic acid, none of it by trichloroacetic acid.

Extracts from two batches of grinding D lost 18.3 and 16.2 per cent of their biuret nitrogen after precipitation with trichloroacetic acid, and 31.2 and 30.8 per cent after precipitation with tungstic acid. Trichloroacetic and tungstic acids removed 15.2 and 34.5 per cent, respectively, of the biuret nitrogen of an extract of grinding E.

More interesting data were obtained from the precipitation of the electrophoretic fractions of these extracts (Table IV). After 24 hours, the fractionation of the extracts from grinding D was barely under way; the larger molecules (precipitated by trichloroacetic acid) had begun to migrate toward the acidic cells while those intermediate in size (precipitated by tungstic acid, but not by trichloroacetic) were still equally distributed.

A longer period of electrophoresis on an extract of grinding E gave more striking results. The largest molecules seemed to be isolectric about pH 3.6 while the intermediate size particles were definitely more alkaline. Approximately equal amounts of biuret nitrogen (not shown) occurred in the first four cells, thus indicating that most of the material had very feeble amphoteric properties (7). Since only about one-third of the biuret nitrogen of the original extract was removed by tungstic acid, two-thirds of it must be accounted for, by small acidic molecules, probably of peptone dimensions.

Polarography

The polarographic studies were initiated by checking diffusion current intensities against concentration (12). Pure cystine solutions in concentrations from $4 \times 10^{-6}M$ to $32 \times 10^{-6}M$, gave a straight line relationship when current was plotted against concentration. An extract of powdered wool, grinding B, that contained about 40 mg./100 ml. of protein nitrogen was electrolyzed, and the waves were graphed directly from galvanometer readings. A sample of the extract was diluted 1:2 and the procedure repeated. The cobalt wave was followed by the characteristic double wave of protein containing cystine, with plateaus at -1.4 and -1.6 volts (13). The wave heights for the two solutions were in the ratio of 1:2 when measured by either the first or second plateau. Repeated duplication of this linearity between concentration and diffusion current established this relation for future studies. Brdicka (13) has shown that this linear function operates only within certain limits of protein concentration, but apparently these limits were not exceeded by any concentrations of the products from wool protein for which our

instrument was standardized. Fig. 1 illustrates the waves and the method used for measuring their heights. Using a single soluble protein, Brdicka (13) employed the change in ratio of the two waves to determine optimum concentrations of buffer components. Buffer concentrations, mercury dropping time and temperature being kept constant, differences in the ratio should therefore indicate differences in the protein examined. From these observations (13), and those of Crossley, *et al.* (14) it may be concluded that: (1) differences in wave heights reflect differences in

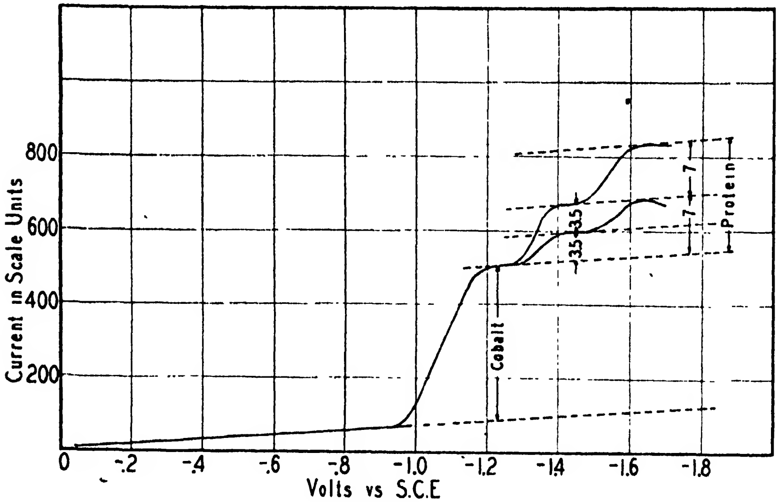

FIG. 1

Method of Measurement and Effect of Dilution on Polarographic Waves of Wool Extracts

Upper curve—1 ml. wool extract (40.0 mg. biuret N per 100 ml.) diluted 1:50 with cobalt buffer.

Lower curve—Same extract, diluted 1:2 before addition of the buffer.

concentration of polarographically active groups present; (2) changes in the double wave structure should be found only when changes in chemical composition or molecular structure are encountered. Extracts of grindings, C, D, E (0.6 g. in 25 ml. of water) from a wool sample were polarographed; the results are shown in Fig. 2. Height ratios (the height of the −1.6 volt wave on the current axis to the height of the −1.4 volt wave measured on the same axis) of the double wave increased, with grinding from 1:3 to 6:11 to 2:3. Wave height was found to be a linear

function of cystine content (determined after hydrolysis) but not directly proportional to it. Total nitrogen content bore no linear relationship to any wave heights. Since the slopes of curves for these graphed relationships were all different, the changes in total and biuret nitrogen and cystine probably occurred at different stages of the grinding process. This was further confirmed by the alteration in the height ratios of the double wave (Fig. 2).

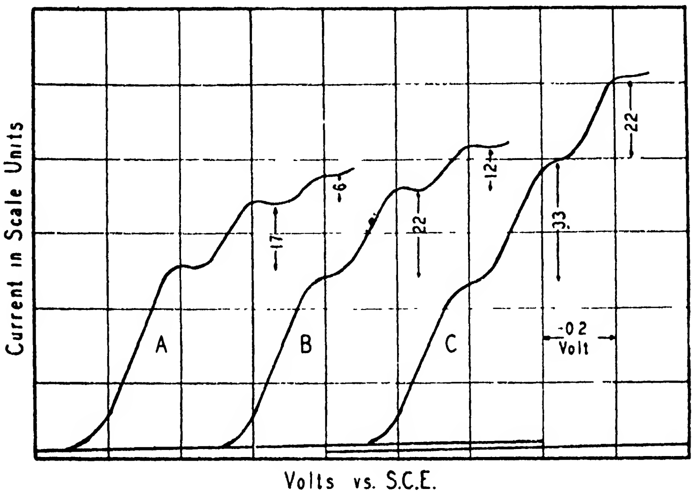

FIG. 2

Polarographic Waves of Extracts of Wool Ground Varying Lengths of Time
A—grinding C, 20.3 mg. biuret N per 100 ml.
B—grinding D, 31.5 mg. biuret N per 100 ml.
C—grinding E, 65.0 mg. biuret N per 100 ml.

If grinding gave rise to a heterogeneous product, each extraction technique might produce an extract of specific chemical composition. Polarographic examination of extracts made by changing the amount of solvent or the number of extractions, supported this hypothesis. Extracts made by three different procedures from samples of grinding E contained variable amounts of biuret nitrogen. Whereas the total wave height increased with the amount of protein present, the double wave ratios varied considerably and were 3:7, 2:1, 2:3 for the three polarograms. The ratio 2:3 was normally encountered in the products of a single extraction. The inverted ratio, 2:1, was unusual.

Polarographic Wave Patterns and Electrophoretic Fractions

"Wave pattern" was a term selected as best fitting a family of current-voltage curves, obtained from the fractions resulting from re-electrophoresis of the contents of a parent cell.

Wave patterns made from an extract of 7.5 g. of wool (grinding D) stirred 3 hours in 300 ml. of water are recorded in Fig. 3 (for economy of

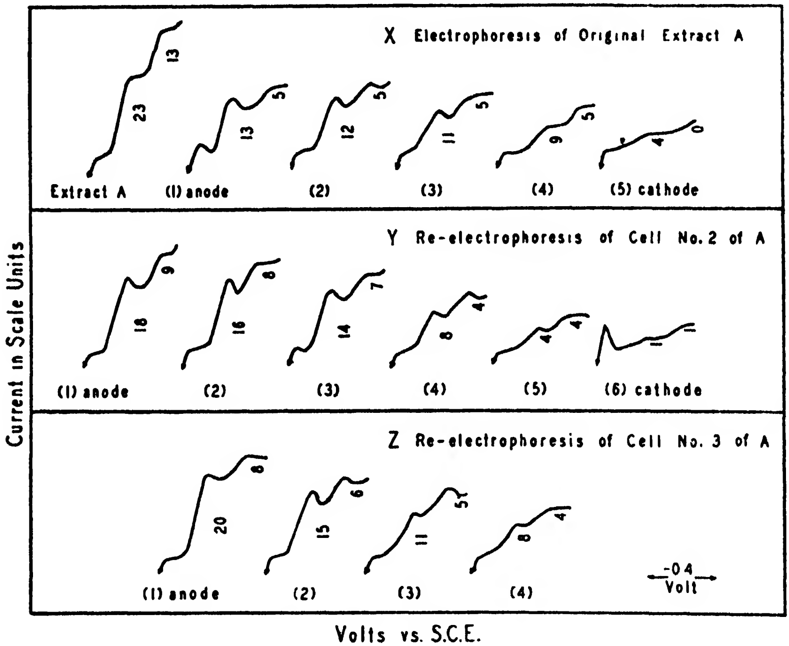

FIG. 3

Wave Patterns of Electrophoretic Fractions

space the entire cobalt wave is not shown). The original extract, A, was separated electrophoretically in the 125 ml. cells; its wave pattern and those of its fractions are shown at X. Cells 2 and 3 were fractionated in the micro set. The resulting wave patterns of the fractions, shown at Y and Z are typical of other polarograms.

In the electrophoretic fractions, wave height was no longer proportional to biuret nitrogen, but diminished progressively with distance from the anode; *e.g.*, cells Z 1, 2, 3, 4 of Fig. 3 had respectively 0.35, 0.31, 0.34, 0.28 mg. biuret nitrogen per ml., but the corresponding wave heights

were 28, 21, 16, 12. Generally, the material in the cells with pH 3 to 4 had the highest biuret nitrogen values and a double wave ratio that approached 1:2.

As in many other fractionation procedures the boundaries between fractions are not sharply defined. Thus the fractions in cells 2 and 3, Fig. 3, had similar double wave structure as did the first four fractions obtained from cell 2, and the 3rd and 4th from cell 3.

The near absence of a double wave from the solutions in cathode cells raised the question of alkali destruction of protein. It has already been shown that the alkaline fractions contained biuret nitrogen, and that the total amount of this in a set of cells remained unchanged during electrophoresis. The dropping mercury electrode presented further proof that protein was not destroyed by the current or the alkali.

Attempts were made to correlate the polarographic data with particle size as indicated by the protein precipitants previously used. Unfortunately the trichloroacetic acid itself altered the polarographic waves. The wave at −1.4 volts for filtrates from tungstic acid precipitation was always lower than for a diluted original extract of equivalent biuret nitrogen content. No plateau could be obtained at −1.6 volts for filtrates, as the current-voltage curves began a very rapid continuous rise for applied voltages more negative than −1.5 volts. In this region galvanometer deflections were wide and erratic; however, controls of tungstic acid in the buffer produced no such effect, and were not different from the buffer alone at the mercury electrode. From this peculiar activity it was concluded that the precipitating agent removed polarographically active material, the activity of which, with respect to the peptid linkage, was relatively greater than that of the average material in the original extract. Since it was known that tungstic acid left behind small molecules, these must have been the ones in which sulfur linkages had suffered greatest destruction at little expense to the peptid bonds.

Comparison of Extracts with Known Soluble Proteins

Solutions of fresh egg white of approximately the same nitrogen concentration as typical wool extracts, were subjected to electrophoresis in the micro cells for 30 hours. The results of the various analyses indicated that protein was concentrating somewhat above a pH of 3.8 in cells 2 and 3. Furthermore, material in these cells, which presumably contained the highest concentrations of egg albumin, gave double polarographic waves with the two plateaus a measurable distance apart.

The solutions of the other cells and the original solution produced a pair of maxima, which according to the accepted method of measurement, resolved into one plateau some distance above the cobalt wave. The fact that egg white contains a globulin fraction (15) offered an explanation applicable to the interpretation of waves obtained from wool extracts. The effect of the globulin was to suppress the double wave character; and its main effect was on the second wave. Presumably the globulin, being less concentrated in cells 2 and 3 than in the others or in the original solution, permitted the appearance of the usual second plateau in the second wave.

These observations suggested that in addition to movement from one cell to another of protein components which varied in polarographic activity, there was like movement of a fraction or fractions which, being either polarographically active or inactive, affected the wave response of the molecules with which they were mixed.

Since preceding studies had indicated that the soluble material from ground wool behaved in some respects like proteoses and peptones, the analogy was extended to polarographic studies on the latter. A solution of Witte's Peptone (0.27 mg. biuret N per ml.) was subjected to electrophoresis in a set of seven micro cells for 24 hours. A definite accumulation of biuret reactive material occurred in the center three cells whose pH readings were 4.5, 5.9, 7.9 respectively. The wave pattern indicated that each cell contained a mixture of substances of varying polarographic activity; the wave heights and changed wave structures were out of proportion to the changes in peptid concentrations. In the original solution and in the acidic cell solutions the second hump of the double wave was replaced by a steep slope thus resembling the behavior of the tungstic acid filtrates of wool extracts. In marked contrast to the latter, continued narrow galvanometer oscillations were observed throughout the entire wave recording. When a solution of proteoses and peptones having no second wave was diluted, a second wave appeared. This indicated the presence of a substance responsible for the slurring of the second wave, a substance more sensitive to dilution than the material responsible for the orthodox double wave. This explanation is in harmony with the hypothesis that tungstic acid removed from solution a wave-producing entity, thereby increasing the mole fraction of the wave slurring entity.

Discussion

The increased acidity of extracts of wool after prolonged grinding is probably due to oxidation of the cystine with the formation of acidic

products (*i.e.*, sulfenic, sulfinic, and sulfonic acids). Although the ratio of biuret nitrogen to total nitrogen of the extracts remained fairly constant, the response to protein precipitants such as acetone and alcohol or isoelectric conditions was consistently negative, suggesting a marked decrease in molecular size.

During electrophoresis and re-electrophoresis of the extracts the fractions containing the highest concentration of biuret and tyrosine nitrogen were isoelectric at a pH of about 3. This would suggest a relatively high concentration of protein fragments to which oxidized sulfur derivatives were attached. As judged by response to protein precipitants these fragments in the acid fractions were larger than those in the fractions with more alkaline isoelectric points. They account for about one-third of the biuret nitrogen in the extracts, the remainder being in non-precipitable fragments of the size of peptones or smaller. These same fractions which accumulate in the electrophoresis cells in the region of pH 3 must also contain fragments with less highly oxidized sulfur linkages including some unchanged cystine since these fractions exhibit a greater polarographic activity than those that separate at a more alkaline pH. The cause of the distortion of the polarographic waves by these smaller fragments which are relatively inactive polarographically remains to be determined.

Perhaps the study of other proteins such as gelatin, peptones, and egg white may be facilitated by the information here obtained since some of their components demonstrate polarographic behavior similar to that of the water-soluble fragments from powdered wool.

Summary

1. Aqueous extracts of wool which had been ground for varying lengths of time in a steel ball mill were studied with respect to the physical and chemical nature of the soluble fraction so produced.

2. Precipitation data showed that only relatively few of the soluble particles were large enough to be precipitated by trichloroacetic acid. Tungstic acid removed less than half the biuret nitrogen from solution.

3. When the soluble components were subjected to electrophoresis several different fractions were obtained. These fractions were characterized by variations in acidity, in biuret and tyrosine nitrogen content, and a change in response to the above protein precipitants.

4. Polarographic studies indicated the heterogeneous nature of the soluble material with a concentration of the polarographically active

material in the fractions in the region of pH 3. These fractions also contained the major portion of the precipitable nitrogen.

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A Simple Method for Blood Sugar

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Introduction

The dinitrosalicylic acid reagent devised by Sumner in 1925 for the estimation of sugar in the urine of normal and diabetic individuals (1) cannot be used directly in the determination of the glucose in blood filtrates by the Folin-Wu method (2), because an appreciable quantity of glucose is oxidized by the oxygen dissolved in the blood filtrate, while some glucose is caramelized by the alkali of the reagent before it has had time to exert a reducing action. We have drawn this conclusion from the results of experiments in which all oxygen was removed from the reactants by the use of a stream of nitrogen gas.

We have discovered that it is possible to obtain quantitative values for blood sugar by first evaporating the Folin-Wu filtrate to dryness in a porcelain evaporating dish and then adding the dinitrosalicylic acid reagent and heating. The two reasons for the effectiveness of this procedure are that the dinitrosalicylic acid reagent, which contains very little dissolved oxygen on account of its high content of Rochelle salt, is the only solution used and that the reagent is not diluted by the addition of blood filtrate, this having evaporated. Consequently the concentration of dinitrosalicylate is high enough to oxidize the glucose before the latter becomes caramelized.

The reaction between glucose and our reagent is extremely rapid. Five minutes of heating on the steam bath are ample. Ten minutes of heating cause no increase in color production. After the 5 minutes of heating, the colored solution is transferred to a 25 ml. volumetric flask, diluted to the mark, mixed, and a reading made in a photoelectric colorimeter. It is also possible to make a reading for the solution in an ordinary colorimeter, with a standard prepared by treating 0.2 mg. of glucose in the same manner as the unknown.

We have found that some Folin-Wu blood filtrates contain enough

acid to occasion a considerable destruction of glucose during the last stages of the evaporation to dryness. This destruction is prevented by adding acetate buffer. The acetic acid all evaporates and the sodium acetate which remains is not alkaline enough to destroy the glucose, nor does it affect the alkalinity of the disnitrosalicylic acid reagent. This reagent contains considerably more sodium hydroxide than is necessary for the reaction with glucose to proceed to completion.

We have demonstrated that with our method it is possible quantitatively to recover glucose which has been added to blood filtrate. It may be considered that the evaporation of blood filtrate to dryness on a steam bath is a time-consuming process. However, ordinarily only 2 ml. of filtrate are needed for the analysis, and the evaporation requires only 10 minutes.

Our dinitrosalicylic acid reagent is probably more specific for glucose than any other reagent in common use. It does not react at all when boiled with cysteine or glutathione, whereas these thiol compounds reduce Fehling's solution and similar copper reagents in the cold. It should be noted, however, that added cysteine causes glucose to give a somewhat greater reduction with the dinitrosalicylic acid reagent than does glucose alone.

Method

Place a Coors No. 000 (6 cm. in diameter) porcelain evaporating dish on a steam bath and pipette into it from 1 to 5 ml. of blood filtrate; usually a 2 ml. quantity is the correct amount. Add 5 drops of 0.5 *M* acetate buffer of pH 5.0 to 5.5. When the liquid has evaporated entirely, add 1 ml. of the new dinitrosalicylic acid reagent. With the fingers, or with tongs, rotate the dish in order to mix the reagent with the dried material. At once cover with a watch glass; continue the heating for 5 minutes. Then wash the reddish brown material into a 25 ml. glass-stoppered volumetric flask. Dilute to the mark, mix, and make a reading on a photoelectric colorimeter, using a green filter. To set the photoelectric colorimeter at the zero point, employ a solution of the dinitrosalicylic acid reagent which has been diluted 25-fold. To obtain the glucose value from the colorimetric reading one must refer to a standard curve which has been prepared by running analyses (the evaporation in the evaporating dish being used as a basis) with 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, and 0.60 mg. of pure glucose and plotting scale A readings against the mg. of glucose. Our scale A readings fot the above values of glucose are given in Table I. A Fisher electrophotometer was used. It must be noted that these values will not apply to other instruments.

Discussion

In Table II the results on oxalated human blood obtained by the Folin-Wu method (2) are compared with those for the Shaffer-Somogyi method (3), in which we deproteinized the material according to Somogyi's earlier technique (4) and used the Shaffer-Somogyi No. 50 copper reagent. The Folin-Wu filtrate was used for our own method. It will be seen that

TABLE I

Glucose *mg.*	Scale A
0.05	6.0
0.10	14.5
0.20	30.0
0.30	44.5
0.40	59.0
0.50	75.5
0.60	87.0

TABLE II

mg. glucose per 100 ml. blood

Experiment No.	Folin-Wu method	Somogyi-Shaffer-Hartmann method	Authors' method
1	132		122
2	87	73	80
3	114	89	95
4	89	67	77
5	78	60	61
6	86	66	65
7	83	75	81
8		196	195
9	150	134	144
10	596	550	550
11	47	40	40
12	100	92	85

our glucose values are always lower than those obtained with the Folin-Wu method and that our values average a little higher than results by the Somogyi-Sheffer-Hartmann method. We consider normal blood sugar values for our method to lie between 60 and 105 mg. of glucose per 100 ml. of blood.

Preparation of Reagent

Place 255 g. of Rochelle salt and 500 ml. of distilled water in a 2-liter-Erlenmeyer-flask and dissolve by heating. Add 8.8 g. of dinitrosalicylic

acid and 588 ml. of 1.25 *N* sodium hydroxide. Continue the heating until the dinitrosalicylic acid is dissolved. Add 7 g. of phenol and 7 g. of anhydrous sodium bisulfite ($Na_2S_2O_5$). Cool and dilute with distilled water to 1400 ml. Five ml. of this reagent should neutralize 20 ml. of 0.1 *N* hydrochloric acid, phenolphthalein being used. The reagent retains its effectiveness for many months if kept stoppered. The 25-fold diluted reagent employed to set the photoelectric colorimeter at the zero point will remain serviceable for several weeks if kept stoppered.

Summary

A simple method for the determination of blood sugar is described. The method consists essentially in evaporating 2 ml. of the Folin-Wu blood filtrate to dryness, heating with 1 ml. of dinitrosalicylic acid reagent, diluting to 25 ml., and making a reading in a photoelectric or in an ordinary colorimeter.

Acknowledgment

We wish to express our gratitude to the Rockefeller Foundation for financial assistance. Our thanks are due to Dr. Michael Somogyi for sending us sugar values corresponding to ml. of thiosulfate for the Shaffer-Somogyi reagent.

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Sulfa Drug Interference in Sugar Determinations*

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Sulfanilamide interference in sugar determinations by certain alkaline copper methods has apparently not been reported previously. This interference is due to the formation of a cuprous-sulfanilamide complex during the reduction process (1). The degree of interference depends largely on the absolute amount of sulfanilamide in the determination since it removes cuprous ion to form the colorless alkali soluble complex. The compound is decomposed in part by acid with the liberation of cuprous ion. For this reason the greatest discrepancies in sugar determinations are found in those methods employing the amount and appearance of cuprous oxide formed as criteria of the sugar content of the test solution; *i.e.*, certain of the qualitative urine sugar methods.

Urine Sugar

During therapy the level of free sulfanilamide in human urine may reach 300 mg. per cent or higher; consequently, false qualitative sugar estimations may be expected especially in urine from diabetics receiving this drug unless care is exercised as to the sugar method used.

In order to establish the degree of interference caused by sulfanilamide, various levels of glucose and of the drug were dissolved in normal urine, or the drug alone was added to diabetic urine. A number of the commonly used qualitative tests were applied to such samples.[1]

* Aided in part by a grant from the General Research Council, Oregon State System of Higher Education.

[1] Somogyi (2) has recently published a rapid and simple method for urine sugar estimations. Since the reagent contains no copper salt, sulfanilamide does not affect the results. Our adaptation of this method to the photoelectric colorimeter has yielded such excellent results that it has been employed throughout this work for control urine sugar determinations. We agree with Somogyi's statement that the "accuracy of the method is great enough to advance it into the rank of adequate quantitative methods."

Representative data are presented in Table I. It can be seen that the Benedict test is in error when the urine contains as much as 150 mg. per cent of free sulfanilamide, but that 300 mg. per cent of the drug does not increase the error. Further, discrepancies occur only in urine of low or intermediate sugar content (1.4% sugar and less). Above this level of sugar the ratio of cuprous oxide formed to cuprous ion removed is apparently large enough to obscure the discrepancy. A greater degree of error

TABLE I

Sulfanilamide Interference in Qualitative Urine Sugar Determinations by the Benedict Method and by the Clinitest

Sample	Glucose per 100 cc. Somogyi[1]	Sulfanilamide per 100 cc.	Benedict Reading	Clinitest Reading
	g.	*mg.*		
2	0.47	0	+	+
		150	trace	−
		300	trace	−
3	0.66	0	++	++
		150	+	+
		300	+	+
4	0.68	0	++	+
		300	+	−
5	1.40	0	+++	++++
		150	++	+++
6	1.38	0	+++	+++
		150	++	+++
7	2.92	0	++++	++++
		150	++++	+++

[1] See footnote page 337.

is seen from these levels of sulfanilamide when the "Clinitest" (Effervescent Products, Inc.) is employed, and the error is also evident at higher sugar levels (up to 3 per cent).

Since the Sheftel test (Eli Lilly and Company) is apparently not affected by as much as 500 mg. per cent sulfanilamide even in urine samples containing small amounts of sugar, data obtained using this method are not included in Table I. No adequate explanation for this is at hand since the method is similar in many respects to the Clinitest.

Certain quantitative urine sugar methods also yield low results when sulfanilamide is present. However, the degree of interference caused by the levels of the drug employed in this study is not of clinical significance, although for meticulous work this must be given consideration.

For instance, a urine sample containing 0.48 per cent glucose by the Shaffer-Hartmann method indicated 0.45, 0.43, 0.41 per cent after dissolving sulfanilamide in the urine to give concentrations of 100, 200, 300 mg. per cent respectively. In the case of the highest drug concentration, the 5 ml. aliquot of the 1–50 dilution used for analysis contained only 0.3 mg. of sulfanilamide. Assuming a ratio of $3Cu^+$ to 2 sulfanilamide in the complex (1) the amount of cuprous ion made unavailable for iodine reduction just accounts for the titration difference found. The same situation has been found in many instances, but only when the weight of the complex present in a determination is about 0.5 mg. or less. When a larger amount of the complex is formed (from a higher sulfa drug concentration) a portion of it, but never all of it, is decomposed on acidification liberating cuprous ion which then reacts. This leads to the supposition that there is an equilibrium point in the acid decomposition of the complex at which a small amount remains as such, and consequently some of the cuprous ion does not react. This mechanism would account for the observed discrepancies.

Any sugar method, then, in which cuprous ion formed during the sugar oxidation, is used in further reactions will be in error when sulfanilamide is present. This applies to the Shaffer-Hartmann and the Folin-Wu methods. An alkaline copper method not in this category is the quantitative urine procedure of Benedict, in which urine is titrated into the copper reagent to a colorless end point.

Sugar methods employing iron reduction, such as the Hagedorn-Jensen and the Folin-Malmros are unaffected by sulfanilamide.

Blood Sugar

The small amounts of sulfanilamide occurring in blood cause little interference. As an example, human blood containing 90 mg. per cent glucose by the Shaffer-Hartmann method, had an apparent value of 85 mg. per cent after adding 25 mg. of sulfanilamide to 100 ml. of the blood.

Discussion

The explanation for sulfanilamide interference in sugar determinations is based essentially on the following observations and reasoning. If 5 ml.

of an aqueous solution containing 100 mg. per cent glucose and 400 mg. per cent sulfanilamide are boiled with 5 ml. of Shaffer-Hartmann reagent, the copper color disappears completely. *No cuprous oxide appears*, but a white crystalline material forms. This is composed of cuprous copper and the sulfa drug. The same result can be obtained with other alkaline copper reagents although more of the glucose-sulfanilamide solution is required for those reagents with higher copper content.

Crystals which appear identical to those formed in the above experiment can be identified microscopically from the reaction mixture after performing the Benedict qualitative urine test or the "Clinitest," providing sugar and sulfanilamide are present.

The interference in the Benedict qualitative test, as an example, may be pictured as follows: glucose reduces cupric ion to cuprous ion, which forms a complex molecule with sulfanilamide. This diminishes the amount of cuprous oxide formed and also alters its appearance since this complex is white and the small amount of dissolved complex is colorless.

Sulfathiazole forms a yellow to orange complex with cuprous ions under the strongly alkaline conditions of the Benedict qualitative urine test and the "Clinitest." For this reason the results are slightly high although in a large number of such tests with varying levels of sugar and this drug in urine the error was never great enough to upgrade the result as much as one plus. With copper solutions of lower alkali content a white crystalline complex is formed (1).

In quantitative sugar determinations by methods such as the Shaffer-Hartmann this drug tends to give high values. The amounts present in urine or blood under normal conditions are insufficient to cause an error of clinical significance. However, as little as 0.5 mg. in a Shaffer-Hartmann blank results in a titration difference of about 0.4 ml. One, 2, 5 mg. result in differences of 0.6, 1, 2+ ml. respectively. Similar differences are noted in the presence of sugar suggesting that the titration difference or error is not related to the reactions of sulfathiazole and cuprous ion. One would rarely find more than 0.3 mg. of this drug in the aliquots of physiological solutions used for analysis. The mechanism involved in this interference is not clear.

Sulfanilamide added to blanks in the amounts mentioned above causes only a tenth as much titration difference as does sulfathiazole. Since *p*-aminobenzoic acid also decreases the amount of iodine available for titration the phenomenon is not peculiar to the sulfa drugs. Since phthalyl sulfathiazole acts similarly a free para amino group must not be essential.

It should be mentioned that in these determinations the starch iodine end point returns on standing.

To avoid interference in urine sugar determinations, the Somogyi (2) method can be used. On the other hand, the "Clinitest" has yielded good results on urine which had been treated with norite and filtered. About 0.5 g. of norite in 5 ml. of urine removes sufficient of the sulfa drug to eliminate the interference. Urine clarified in this manner gives atypical colors when used with the Benedict test just as pure glucose solution does.

In general other commonly used sulfa drugs, because of their insolubility, do not occur in urine in sufficient quantity to warrant further comment.

Summary

1. Sulfanilamide, at levels occurring in urine of patients on therapy with this drug, causes low readings in certain qualitative urine sugar methods.

2. Some quantitative sugar methods give low results also when sulfanilamide is present.

3. Sulfathiazole has little effect on qualitative urine sugar methods but causes large errors in certain quantitative procedures. The direction of error is opposite to that caused by sulfanilamide.

4. In general other sulfa drugs are found in urine at levels low enough to offer little or no interference.

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Copper Complexes of Sulfanilamide and Sulfathiazole*

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Introduction

Some of the commonly used sulfa drugs have been found to interfere in the determination of glucose by certain of the alkaline copper methods (1). It has been demonstrated that copper complexes of these drugs are formed during sugar determinations and are in part responsible for the errors encountered. The following deals with the preparation and some of the properties of the sulfanilamide copper complex and the sulfathiazole copper complex.

Sulfanilamide Copper Complex

This compound can be prepared by dissolving a reducing sugar and sulfanilamide in an alkaline solution of copper sulfate containing tartrate or citrate. Any one of the commonly used alkaline copper sugar reagents is satisfactory. One method of preparation is as follows: to 5 ml. of Shaffer-Hartmann reagent are added 5 ml. of an aqueous solution containing 100 mg. per cent glucose and 400 mg. per cent sulfanilamide. On heating this mixture in a boiling water bath or over a free flame the blue color disappears completely and a white crystalline material precipitates. No cuprous oxide appears. In the absence of reducing sugar no perceptible reaction occurs. At room temperature the reaction requires several hours.

The crystalline material is readily purified by washing several times with cold water. On drying in a vacuum desiccator the crystals darken slightly and continue to discolor with time. The compound is stable and quite soluble in alkali, but insoluble and unstable in the common organic

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solvents. It is insoluble in water but suspends readily, and the addition of strong acid causes immediate decomposition with the liberation of cuprous oxide.

After removal of the copper by hydrogen sulfide, sulfanilamide can be extracted with ethyl acetate. Following purification, the compound so obtained has a melting point of 162.5° C., which is unchanged upon mixing with pure sulfanilamide. Decomposition commences at about 200° C. Spectographic analysis shows the absence of metals other than copper.

Iodometric titration indicates that more than 95 per cent of the copper of the sulfanilamide complex is present in the cuprous state. It is felt that the remainder is also present in this state, and that the low recovery is due to unknown reactions during the determination.

Analysis of the copper complex indicates 3 atoms of copper, 4 atoms of nitrogen, and 2 atoms of sulfur per molecule.

Calculated for $(C_6H_8N_2SO_2)_2Cu_3$:	Cu 33.65%,	N 10.47%,	S 11.99%.
Calculated for $(C_6H_8N_2SO_2)_2Cu_3(OH)_2$:	Cu 33.5%,	N 9.8%,	S 11.25%.
Found:	Cu 33.3%,	N 9.6%,	S 11.1%.

The data are uniformly high for a compound containing 2 molecules of sulfanilamide and 3 atoms of copper. Since no appreciable weight loss was found on drying the compound in vacuo at 100° C., in an Abderhalden dryer over P_2O_5 the presence of water of crystallization is unlikely. The analytical values agree with the formula containing two hydroxyl groups.

Sulfathiazole Copper Complex

Sulfathiazole reacts with cuprous ion under the conditions previously described, but forms a white crystalline compound only if the alkalinity of the reaction mixture is low. With increasing alkalinity the color of the crystals increases from yellow to orange. The various colored crystals appear identical microscopically.

The white crystalline complex can be purified by several washings with water. This compound is stable in alkaline solution, but less soluble than the sulfanilamide complex. It is rather soluble in dilute acid but decomposes on standing. Strong sulfuric acid liberates cuprous oxide; hydrochloric acid dissolves the compound with the development of a green color. No discoloration occurs on long standing in the dry state. Decomposition commences at about 300° C. Sulfathiazole can be recovered

from the complex by methods similar to those used in the case of sulfanilamide and melting point determinations indicate the purity of the compound. Attempts to determine the state of copper in the sulfathiazole complex by iodometric titration have been unsuccessful.

Analysis of the complex indicates 1 atom of copper, 3 atoms of nitrogen, 2 atoms of sulfur per molecule. A ratio formula meeting these requirements is (sulfathiazole) Cu.

Calculated for $(C_9H_9N_3S_2O_2)Cu$:	Cu 19.99%, N 13.2%, S 20.16%.
Found:	Cu 19.9%, N 13.1%, S 20.0%.

Sulfur was determined by a modification of the method of Gunther, Beier, and LaDue (2). Nitrogen was estimated by the Rinehart, Grondahl, and West (3) modification of the micro Kjeldahl method. After wet digestion copper was estimated iodometrically (4).

Comment

A number of sulfa drugs have been found to form copper complexes. Succinyl sulfathiazole, acetyl sulfanilamide, and phthalyl sulfathiazole, however, do not react under the conditions employed. Thus it appears that a free amino group is essential to the reaction. *p*-Aminobenzoic acid does not react under these conditions.

Attempts to form such complexes by treating sulfanilamide with cuprous oxide under various conditions have not been successful.

In Northey's extensive review (5) of sulfanilamide derivatives no mention is found of a copper sulfanilamide compound comparable to the one described here, nor has a review of the literature disclosed any reports of compounds similar to either of those described above.

Summary

Crystalline complexes of cuprous copper and sulfanilamide and of cuprous copper and sulfathiazole have been prepared by heating a mixture of one of the drugs and a reducing sugar with Shaffer-Hartmann or other alkaline copper sugar reagents.

Both of these complexes are stable in alkaline solution, but are insoluble and unstable in the common organic solvents and in water. When suspended in water they undergo immediate decomposition with the liberation of cuprous oxide on the addition of strong acid. The white sulfanilamide complex darkens rapidly after drying but the sulfathiazole complex remains white in the dry state. The latter complex can be pre-

pared in white, yellow, or orange colored crystals, all of which appear identical microscopically.

On the basis of analytical data the following formulas are proposed:

(a) $(C_6H_8N_2SO_2)_2Cu_3(OH)_2$ for the sulfanilamide complex,

(b) $(C_9H_9N_3S_2O_2)Cu$ for the sulfathiazole complex.

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The Reaction of Ammonia in the Van Slyke Volumetric Amino Nitrogen Method

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Introduction

By the use of the iodide modification (1) of the Van Slyke volumetric method (2, 3) and a 10 minute reaction period, theoretical values can be obtained for α-amino nitrogen in all amino acids except tryptophan and for α-amino plus ϵ-amino nitrogen in the case of lysine (4–7).[1]

The Van Slyke volumetric method is used frequently to measure the rate of hydrolysis of peptide bonds in proteins. However, the effect of the presence in protein hydrolyzates of the ammonia formed during hydrolysis upon the accuracy of the Van Slyke procedure in indicating the true amino nitrogen values of such hydrolyzates has not been thoroughly investigated although experiments on the extent of the reaction of ammonium sulfate solutions with nitrous acid have been reported by Van Slyke (10). Furthermore, no data have appeared to indicate to what extent the reaction of ammonia in the Van Slyke method is influenced by the initial ammonia concentration or by the presence of α-amino groups.

[1] Data to show that tyrosine yields amino nitrogen values which are considerably higher (30 to 70 per cent) than theory in the Van Slyke manometric method (15 minute reaction period), apparently because of the action of visible light on the reaction mixture, have been published recently by Fraenkel-Conrat (8) who also states that a similar though "somewhat less pronounced effect of light was observed in the volumetric apparatus (15 minutes)." This effect has been investigated, and the results, which are reported elsewhere (9), reveal that the error due to the action of intense visible light on the tyrosine-nitrous-acid mixture in the Van Slyke volumetric apparatus is of the order of 6 per cent. In diffused daylight the error is approximately 2 per cent.

The results of the present investigation indicate that the extent of reaction of ammonia in the Van Slyke volumetric method, as it is usually employed and when the initial ammonia concentration lies within the range normally found in protein hydrolyzates, is significant and measurable. The fraction of the ammonia that reacts is independent of the initial concentration of ammonia, but depends upon the length of the reaction period, the characteristics of the apparatus used, and the presence or absence of iodide. An ammonia correction factor can be determined readily for any given apparatus and experimental conditions. This factor can be applied with a satisfactory degree of accuracy to Van Slyke values obtained on protein hydrolyzates and thus "true" amino nitrogen values can be obtained, without necessitating the removal of ammonia from the hydrolyzate.

Apparatus

Three types of Van Slyke volumetric apparatus, manufactured by two different laboratory equipment companies, were used in this investigation. They are hereinafter referred to as microapparatus-A, microapparatus-B, and macroapparatus. The three types of apparatus had the following distinguishing structural characteristics (outside diameter of the reaction chamber and length of the shaking stroke at the calibration mark on the reaction chamber, respectively): Microapparatus-A, 18.0 and 20 mm.; microapparatus-B, 19.5 and 33 mm.; macroapparatus, 32.0 and 33 mm. Both types of microapparatus were equipped with a 3.0 ml. gas burette graduated in 0.01 ml. divisions; the macroapparatus was equipped with a 40.0 ml. gas burette graduated in 0.10 ml. divisions; Hempel pipettes of identical size and construction were used with each apparatus.

Reagents

The reagents used were: Sodium nitrite, ACS grade, 300 g. per liter; acetic acid, glacial, CP grade; potassium iodide, ACS grade, 5.0 g. per liter of sodium nitrite solution, as recommended by Kendrick and Hanke (1); alkaline permanganate, 25 g. potassium hydroxide, CP grade, and 50 g. potassium permanganate, CP grade, per liter; and capryl alcohol, unpurified (The Resinous Products Co.).

Procedure

In both types of microapparatus, accurately pipetted 2.0 ml. samples of the test solutions were used throughout the investigation, and each sample was carefully

rinsed into the reaction chamber by means of exactly 1.0 ml. of water. Blanks were determined on 3.0 ml. of water. Approximately 0.2 ml. of capryl alcohol was used in each determination and blank. In the macroapparatus, accurately pipetted 10.0 ml. portions of test solutions were used throughout, and each sample was rinsed into the reaction chamber by means of exactly 2.0 ml. of water. Blanks were determined using 12.0 ml. of water. Approximately 0.2 to 0.4 ml. of capryl alcohol was used in each determination and blank.

In each apparatus the shaking rate was adjusted to give the most efficient mixing of the contents of the reaction chamber. Because of structural differences, the optimum shaking rates for the three types of apparatus differed, but careful control of the shaking rate was exercised throughout the experiments in order to minimize possible effects due to this variable. The shaking rate in microapparatus-A varied between 260 and 320 strokes per minute; in microapparatus-B, 230 to 275 per minute; and in the macroapparatus, 135 to 160 per minute.

Results

Reaction of Ammonia in Solutions of Alanine Plus Ammonia. In the first series of experiments amino nitrogen determinations were conducted in all three types of apparatus on solutions containing a constant amount (0.5 mg. per ml.) of amino nitrogen as *dl*-alanine[2] and varying amounts of ammonia nitrogen (0.16 to 0.48 mg. per ml.) as ammonium chloride. Ammonia concentrations were selected to cover the range which would be found in complete hydrolyzates of 1 per cent solutions of proteins yielding 10 to 30 per cent of their total nitrogen as ammonia. Sodium nitrite solution containing potassium iodide was used in both the 5 and 15 minute reaction periods. Determinations were made in quadruplicate on each sample, and the data are presented as averages in Table I.

If consideration is first given to the average values for the percentage of ammonia which reacts in a given reaction period, it is seen that the extent of reaction of ammonia is quite dependent upon the type of apparatus used. Thus, approximately 21 per cent of the ammonia reacts in 5 minutes in microapparatus-A, while approximately 31 and 47 per cent of the ammonia react in the same period in microapparatus-B and in the macroapparatus, respectively. Due to structural differences in the apparatus, it is apparent that opportunity for the reaction of ammonia is much greater in the macroapparatus than in either of the two types of microapparatus. With regard to the latter, microapparatus-B permits more rapid reaction of ammonia than does microapparatus-A. These observations explain the relatively large variation (±2 to ±4 per cent) in the values obtained on solutions containing ammonia as contrasted

[2] Analysis calculated: C 40.50, H 7.81, N 15.72. Found: C 40.50, H 7.98, N 15.71.

TABLE I

Extent of Reaction of Ammonia in the Van Slyke Volumetric Amino Nitrogen Apparatus in Solutions Containing Varying Amounts of Ammonia and a Constant Amount of Amino Nitrogen

Potassium iodide, 0.5 per cent, present in the nitrite solution; temperature, 25 to 28°C.

Reaction Period	Sample No.	Nitrogen Found ($NH_2 + NH_3$)[1]	Ammonia Theory	Nitrogen Found[2]	Ammonia Reacted
mins.		*mg. per ml.*	*mg. per ml.*		*per cent*
		Microapparatus-A			
5	G164	0.532	0.160	0.032	20.0
5	G165	0.565	0.320	0.065	20.3
5	G167	0.603	0.480	0.103	21.5
Average					20.6 ± 1.8[3]
15	G164	0.572	0.160	0.072	45.0
15	G165	0.655	0.320	0.155	48.4
15	G167	0.760	0.480	0.260	54.2
Average					49.2 ± 3.6
		Microapparatus-B			
5	G164	0.548	0.160	0.048	30.0
5	G165	0.600	0.320	0.100	31.2
5	G167	0.648	0.480	0.148	30.8
Average					30.7 ± 3.4
15	G164	0.582	0.160	0.082	51.2
15	G165	0.673	0.320	0.173	54.1
15	G167	0.752	0.480	0.252	52.5
Average					52.6 ± 3.9
		Macroapparatus			
5	G164	0.576	0.160	0.076	47.5
5	G165	0.657	0.320	0.157	49.0
5	G167	0.720	0.480	0.220	45.8
Average					47.4 ± 1.6
15	G164	0.614	0.160	0.114	71.3
15	G165	0.730	0.320	0.230	71.9
15	G167	0.839	0.480	0.339	70.6
Average					71.3 ± 2.3

[1] Each sample contained 0.500 mg. per ml. of amino nitrogen as alanine.

[2] Figures in this column were obtained by subtracting 0.500 mg. (the theoretical value for the amino nitrogen present) from the amino plus ammonia nitrogen values shown in Column 3.

[3] Each average and standard deviation calculated from 12 determinations.

with the small variation (±0.3 to ±1.1 per cent) in the values obtained on solutions of alanine alone (Table II).

The results also indicate that in each apparatus and reaction period, with the possible exception of the 15 minute reaction period in microapparatus-A, the fraction of the ammonia that reacts is independent of the initial ammonia concentration within the limits (0.16 to 0.48 mg. ammonia nitrogen per ml.) which were thoroughly investigated. Determinations made in the macroapparatus under the same conditions on solutions containing 0.5 mg. amino nitrogen and 0.08 mg. ammonia nitrogen per ml. indicate that the extent of reaction of ammonia (48.5 per cent in 5 minutes) is essentially the same as that found at higher ammonia concentrations. These figures have not been included in Table I, nor

TABLE II

Analysis of dl-Alanine in the Van Slyke Volumetric Apparatus

0.5 mg. amino nitrogen per ml.; potassium iodide, 0.5 per cent, present in the nitrite solution; temperature, 25 to 28°C.

Apparatus	Reaction Period *mins.*	Amino Nitrogen Found *mg.*	Amino Nitrogen, *Per cent of Theory*
Micro-A	5	0.486	97.3 ±1.1[1]
	15	0.498	99.6 ±0.4
Micro-B	5	0.495	99.0 ±0.3
	15	0.506	101.2 ±0.3
Macro	5	0.506	101.2 ±0.2
	15	0.504	100.8 ±0.4

[1] Each average and standard deviation calculated from 4 determinations.

have they been used in computing the averages. Furthermore, no attempt was made to determine the extent of reaction of ammonia in either type of microapparatus on solutions containing ammonia in lower concentration than those indicated in Table I, because it is obvious that at very small ammonia concentrations the percentage variation between the experimentally determined values would be exceedingly high. For example, in the determinations conducted in microapparatus-B (Table I), where the gas burette was read to the nearest 0.01 ml., this volume of gas corresponds to approximately 0.006 mg. of amino or ammonia nitrogen. If the amount of amino or ammonia nitrogen found is only 0.048 mg., as was the case with sample No. G 164, then duplicate determinations which check within 0.01 ml. will deviate by as much as 12 per cent. If

the initial ammonia concentration were reduced below 0.16 mg. ammonia nitrogen per ml., then correspondingly higher deviations would be expected. Determinations were not carried out in which reaction periods were less than 5 minutes, inasmuch as the amount of ammonia that reacts, and hence the amount of nitrogen to be measured, becomes smaller as the reaction period is shortened. In view of the data presented, however, it seems reasonable to conclude that, in the presence of alanine and potassium iodide, the fraction of ammonia that reacts in the Van Slyke volumetric procedure is independent of the initial ammonia concentration.

TABLE III

Extent of Reaction of Ammonia in the Van Slyke Volumetric Amino Nitrogen Apparatus in a Solution Containing Both Amino and Ammonia Nitrogen

No potassium iodide present in nitrite solution; temperature, 24 to 27°C.; 15 minute reaction period; sample No. G 164 containing per ml. 0.5 mg. of amino nitrogen as alanine and 0.16 mg. ammonia nitrogen as ammonium chloride.

Apparatus	Nitrogen Found (NH_2 + NH_3)	Ammonia Nitrogen Found[1]	Ammonia Reacted
	mg. per ml.	*mg. per ml.*	*per cent*
Micro-A	0.600	0.100	62.5 ±3.1[2]
Micro-B	0.595	0.095	59.4 ±3.0
Macro	0.630	0.130	81.2 ±2.5

[1] Figures in this column were obtained by subtracting 0.500 mg. (the theoretical value for the amino nitrogen present) from the amino plus ammonia nitrogen values given in Column 2.

[2] Each average and standard deviation calculated from 4 determinations.

Reaction of Ammonia in Solutions of Alanine Plus Ammonia in the Absence of Iodide. Van Slyke determinations, in the absence of potassium iodide, were made in quadruplicate with each apparatus on sample No. G 164 (0.5 mg. amino nitrogen as alanine and 0.16 mg. ammonia nitrogen as ammonium chloride per ml.) employing a 15 minute reaction period. The results are given in Table III where it can be seen that a considerably larger fraction of the ammonia reacts when no iodide is present than when sodium nitrite solution containing 0.5 per cent potassium iodide is used (Table I). These results indicate that potassium iodide decreases the rate of reaction of ammonia with nitrous acid to yield nitrogen. It will be recalled that glycine and cystine react with nitrous acid to yield higher than theoretical amounts of nitrogen and that the presence of

iodide in the reaction mixture reduces the values obtained with these two amino acids to theoretical. It cannot be stated, however, that the reaction of ammonia with nitrous acid is analagous to that of glycine and cystine with the same reagent since in the experiments reported here the ammonia-nitrous-acid reaction was never carried to completion. Nevertheless, both the action of iodide in retarding the rate of reaction of ammonia with nitrous acid and its effectiveness in preventing the

TABLE IV

Extent of Reaction of Ammonia in the Van Slyke Volumetric Amino Nitrogen Apparatus in Solutions of Ammonium Chloride

Potassium iodide, 0.5 per cent, present in the nitrite solution; temperature, 24.5 to 28°C.; reaction period, 15 minutes

Apparatus	Sample No.	Ammonia Nitrogen Theory	Ammonia Nitrogen Found	Ammonia Reacted
		mg. per ml.		*per cent*
Micro-A	G169	0.160	0.074	46.2
	G170	0.320	0.143	44.7
	G171	0.480	0.225	46.9
Average				45.9 ±2.6[1]
Micro-B	G169	0.160	0.080	50.0
	G170	0.320	0.164	51.4
	G171	0.480	0.280	58.3
Average				53.2 ±5.9
Macro	G169	0.160	0.121	75.6
	G170	0.320	0.242	75.6
	G171	0.480	0.354	73.8
Average				75.0 ±1.8

[1] Each average and standard deviation calculated from 6 determinations.

formation of extra nitrogen from glycine and cystine in the same reaction may be significant in the ultimate elucidation of the mechanism of these reactions.

Reaction of Ammonia in Solutions of Ammonium Chloride. In Table IV are given the results of Van Slyke determinations which were made in duplicate with each apparatus on solutions of pure ammonium chloride. Sodium nitrite solution containing potassium iodide was used in each case and a 15 minute reaction period was employed. No significant difference was found between the average values given in Table IV and those given for the 15 minute reaction period in Table I. It is evident,

therefore, that the extent of the reaction of ammonia in the Van Slyke volumetric method is not significantly influenced by the presence of amino groups in the form of alanine.

Reaction of Ammonia in a Protein Hydrolyzate Plus Ammonia. A sample of peanut protein was completely hydrolyzed by refluxing with 5.8 *N*

TABLE V

Extent of Reaction of Ammonia in the Van Slyke Volumetric Amino Nitrogen Apparatus in a Peanut Protein Hydrolyzate Containing Known Amounts of Added Ammonia

Potassium iodide, 0.5 per cent, present in the nitrite solution; temperature, 24 to 27°C.; 15 minute reaction period.

Apparatus	Sample No.	Nitrogen Found (NH_2 + NH_3)[1] *mg. per ml.*	Ammonia Nitrogen Theory *mg. per ml.*	Ammonia Nitrogen Found[2] *mg. per ml.*	Ammonia Reacted *per cent*
Micro-A	G175	0.582	0.176	0.078	44.3
	G176	0.644	0.336	0.140	41.7
	G177	0.759	0.496	0.255	51.4
Average					45.8 ±5.2
Micro-B	G175	0.570	0.176	0.074	42.0
	G176	0.654	0.336	0.158	47.0
	G177	0.726	0.496	0.230	46.4
Average					45.1 ±2.1
Macro	G175	0.633	0.176	0.127	72.2
	G176	0.744	0.336	0.238	70.9
	G177	0.851	0.496	0.345	69.6
Average					70.9 ±1.3

[1] The average amino nitrogen values, determined on the ammonia-free hydrolyzate, were as follows for each apparatus: Micro-A, 0.504; Micro-B, 0.496; Macro, 0.506 mg. amino nitrogen per ml.

[2] The figures in this column were obtained by subtracting the determined values in each apparatus for the amino nitrogen present (given in Footnote 1 above) from the corresponding amino plus ammonia nitrogen values given in Column 3.

hydrochloric acid and the ammonia-free solution was acidified, and known amounts of ammonium chloride were added to prepare solutions for Van Slyke analysis. The results are given in Table V. Determinations were made in duplicate; potassium iodide was present in each case; and a 15 minute reaction period was employed. The values given in Table V

are slightly lower than those given for the 15 minute reaction period in Tables I and IV, but in view of the variations indicated, the differences are not significant. It appears, therefore, that the extent of reaction of ammonia in the Van Slyke volumetric method is not significantly influenced by the presence of the amino acids and other possible products formed during the hydrolysis of peanut protein.

Comparison of Amino Nitrogen Value Calculated from the Van Slyke Amino-Plus-Ammonia Nitrogen Value for a Complete Protein Hydrolyzate with Amino Nitrogen Value Determined Directly on the Same Hydrolyzate After Removal of Ammonia. From the results given so far it is evident that the extent of reaction of ammonia in the Van Slyke volumetric method for a given apparatus and set of experimental conditions, is independent of the ammonia concentration and of the presence (or nature of) amino compounds. It thus becomes possible to calculate the "true" amino nitrogen value of a protein hydrolyzate at any stage of hydrolysis. This is accomplished by first determining the ammonia nitrogen and the Van Slyke values for the hydrolyzate and then subtracting from the Van Slyke value that fraction of the total ammonia nitrogen which is known to react under the conditions used. As has been pointed out in the foregoing sections, however, the ammonia correction factor must be determined for the apparatus used under the actual conditions employed for obtaining the Van Slyke value on the hydrolyzate. This can be done quite simply by determining the percentage of ammonia that reacts under the conditions desired, using a single sample of ammonium chloride solution which has an ammonia concentration within the desired range. The average value obtained from several determinations made on this sample will provide a sufficiently accurate factor for the correction of Van Slyke values on hydrolyzates.

The application of the ammonia correction and the order of accuracy of the correction are illustrated in Table VI. A complete protein hydrolyzate was selected for this experiment because after hydrolysis is complete no significant change in amino nitrogen would be expected to result from the treatments employed to remove ammonia. A 5.0 g. sample of peanut protein was completely hydrolyzed by refluxing with 250 ml. of 5.8 *N* hydrochloric acid for 24 hours. The hydrolyzate was adjusted to pH 1.4 and diluted to 500 ml. Ammonia nitrogen, by the MgO-steam-distillation procedure, and amino-plus-ammonia nitrogen by the Van Slyke volumetric method, were determined on this solution (Columns 2 and 5, respectively, Table VI). A 250 ml. portion of the solution was adjusted

to pH 10.0 by adding sodium hydroxide, and the ammonia was removed by passing live steam through the solution for 7 minutes. The ammonia-free solution was acidified to pH 2.5 and was distilled *in vacuo* to approximately 200 ml. after which it was transferred quantitatively to a 250 ml. volumetric flask and diluted to volume. Van Slyke determinations were then made on the ammonia-free hydrolyzate (Column 6, Table VI). The ammonia correction factor for each apparatus (given in Table V for a similar hydrolyzate containing known amounts of ammonia) was

TABLE VI

Comparison of Amino Nitrogen Values Calculated From the Van Slyke Amino Plus Ammonia Nitrogen Values For a Peanut Protein Hydrolyzate With Amino Nitrogen Values Determined Directly on the Same Hydrolyzate After the Removal of the Ammonia

Temperature, 24.5 to 27°C.; potassium iodide, 0.5 per cent, present in the nitrite solution; 15 minute reaction period

Apparatus	Ammonia Nitrogen in Hydrolyzate	Fraction of Ammonia Nitrogen Which Reacts[1]		Amino + Ammonia Nitrogen in Hydrolyzate	Amino Nitrogen in Ammonia-free Hydrolyzate	Calculated Amino Nitrogen in Hydrolyzate[2]
	mg. per ml.	*per cent*	*mg. per ml.*	*mg. per ml.*	*mg. per ml.*	*mg. per ml.*
Micro-A...	0.172	45.8	0.079	1.072	1.01	0.99
Micro-B .	0.172	45.1	0.078	1.102	0.99	1.02
Macro....	0.172	70.9	0.122	1.172	1.01	1.05

[1] The per cent values used are the averages given in Table V.

[2] Figures in this column were obtained by subtracting the number of mg. of ammonia nitrogen which will react in the Van Slyke method (Column 4) from the amino plus ammonia nitrogen values on the hydrolyzate (Column 5).

used to correct the amino-plus-ammonia nitrogen values to give the values shown in the last column of Table VI.

It is evident that the calculated "true" amino nitrogen values and the amino nitrogen values determined directly on the ammonia-free hydrolyzate are very closely similar, indicating that the method of correction employed is a reliable means for obtaining amino nitrogen values on hydrolyzates which contain ammonia.

Other Applications of the Ammonia Correction Procedure. An example of the usefulness of the ammonia correction procedure in interpreting Van Slyke amino nitrogen data on protein hydrolyzates is illustrated in

Table VII. In this case, three 0.5 N hydrochloric acid hydrolyzates of peanut protein were analyzed in the two types of microapparatus, and a different reaction period was employed with each apparatus. A comparison of the actual Van Slyke values for the 5 and 15 minute reaction periods, given in Column 7, indicates that higher values are obtained in 15 than in 5 minutes with each hydrolyzate. This might be taken to indicate that more amino nitrogen (possibly that of the unhydrolyzed protein) reacts in 15 minutes than in 5 minutes, but actually, as the corrected values in the last column show conclusively, the amount of amino nitrogen that reacts during each reaction period is the same. In

TABLE VII

Calculation of "True" Amino Nitrogen Values From Data Obtained During the Hydrolysis of Peanut Protein by Refluxing With 0.5 N Hydrochloric Acid

Length of Hydrolysis	Apparatus	Reaction Period	Ammonia Nitrogen in Hydrolyzate		Amino + Ammonia Nitrogen in Hydrolyzate (Van Slyke Values)		Amino Nitrogen in Hydrolyzate (Corr.[1] Van Slyke Value)	
hours		*mins.*	*mg. per ml.*	*per cent of total-N*	*mg. per ml.*	*per cent of total-N*	*mg. per ml.*	*per cent of total-N*
1	Micro-A	5	0.188	11.9	0.301	18.7	0.262	16.4
2	"	5	0.206	13.1	0.506	31.4	0.462	29.0
3	"	5	0.207	13.1	0.578	35.9	0.535	33.4
1	Micro-B	15	0.188	11.9	0.341	21.2	0.256	16.0
2	"	15	0.206	13.1	0.565	35.1	0.472	29.5
3	"	15	0.207	13.1	0.631	39.2	0.538	33.6

[1] Correction factors for ammonia: Microapparatus-A, 5 minute reaction period, 20.6 per cent (Table I); Microapparatus-B, 15 minute reaction period, 45.1 per cent (Table V).

other words, each of the determined values for amino nitrogen in this series of determinations is in error, depending upon the amount of ammonia that reacts, and this in turn is dependent upon the amount of ammonia present, the type of apparatus used for the determination, and the reaction period employed.

Another example of the applicability of the ammonia correction procedure is illustrated in Table VIII. The results given emphasize the necessity for applying a correction of this sort to Van Slyke values obtained for the purpose of determining accurately the rate and extent of hydrolysis of peptide bonds in proteins. In this experiment separate

samples of peanut protein were hydrolyzed in 0.5 *N* sodium hydroxide at 80°C. for various periods. Each hydrolysis was carried out in a closed vessel, and all of the hydrolyzates were acidified to approximately pH 4.5 before the vessels were opened in order to prevent loss of ammonia during and after hydrolysis. It can be readily seen by comparing the determined values (Column 5) with the corrected values (Column 7) that the presence of ammonia, if ignored, may constitute a serious source of error in investigations of this type.

TABLE VIII

Calculation of "True" Amino Nitrogen Values From Data Obtained During the Hydrolysis of Peanut Protein by 0.5 N Sodium Hydroxide at 80°C.

Microapparatus-A used; potassium iodide, 0.5 per cent, present in nitrite solution; 15 minute reaction period

Length of Hydrolysis	Ammonia Nitrogen in Hydrolyzate		Amino + Ammonia Nitrogen in Hydrolyzate (Van Slyke Value)		Amino Nitrogen in Hydrolyzate (Corr. Van Slyke Value)[1]	
hours	*mg. per ml.*	*per cent of total-N*	*mg. per ml.*	*per cent of total-N*	*mg. per ml.*	*per cent of total-N*
0	0.000	0.0	0.028	1.75	0.028	1.75
1	0.149	9.34	0.227	14.18	0.159	9.94
3	0.190	11.89	0.315	19.67	0.228	14.25
5	0.206	12.86	0.344	21.50	0.250	15.62
8	0.218	13.65	0.433	27.09	0.333	20.81
16	0.275	17.17	0.570	35.66	0.444	27.75
24	0.282	17.63	0.606	37.84	0.477	29.81

[1] Corrected amino nitrogen values were obtained by subtracting 45.8 per cent (see Table V) of the ammonia nitrogen values (Column 2) from the amino plus ammonia nitrogen values (Column 4).

Kinetics of the Reaction of Ammonia with Nitrous Acid. Under conditions where the reaction of ammonia with nitrous acid can proceed at the maximum rate for a given temperature, it should be possible to determine the order of the reaction and to calculate a rate constant. When Van Slyke's data (10) for the reaction of high concentrations of ammonia in the volumetric apparatus in the absence of iodide are recalculated, and a graph is constructed by plotting the logarithm of the concentration of unreacted ammonia against the time of reaction, a straight line is obtained. This fact indicates that the reaction satisfies the requirements of a unimolecular reaction. Sufficient data have not been obtained in the present investigation to permit calculation of reac-

tion constants. Nevertheless, the data of Van Slyke and the conclusion, also reached in this paper, that the fraction of the ammonia that reacts in a given time is independent of the initial ammonia concentration, make it appear that the reaction of ammonia with nitrous acid, in the absence of iodide, is a unimolecular reaction. The kinetics of the reaction of ammonia with nitrous acid both in the presence and in the absence of iodide is being investigated further.

Summary

The extent of reaction of ammonia with nitrous acid in the Van Slyke volumetric amino nitrogen method, in cases where the ammonia concentration lies within the range normally found in protein hydrolyzates, is measurable and significant.

The fraction of the ammonia that reacts is independent of the initial concentration of ammonia and of the presence or nature of amino compounds, but it does depend upon the length of the reaction period and the structural characteristics of the apparatus used.

Considerably more ammonia reacts in a given period of time when iodide is absent than when potassium iodide is present in the reaction mixture.

An ammonia correction factor can be determined for any given apparatus and experimental conditions, and this factor can be applied to Van Slyke values obtained on protein hydrolyzates for the purpose of calculating "true" amino nitrogen values.

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On Lactic Acid Metabolism in Propionic Acid Bacteria and the Problem of Oxido-Reduction in the System Fatty-Hydroxy-Keto Acid[1]

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Introduction

In the fermentative metabolism of microorganisms and animal tissues, lactate is commonly the final reduction product of pyruvate. Propionic acid bacteria, however, are able to cause the more complete reduction of pyruvate to propionate. Since lactate is also readily converted into propionate by these bacteria, it has generally been assumed that lactate is an intermediate in the reduction of pyruvate, as follows:

$$CH_3\cdot CO\cdot COOH \xrightarrow{+2H} CH_3\cdot CH(OH)\cdot COOH \xrightarrow{+2H}$$
$$CH_3\cdot CH_2\cdot COOH + H_2O.$$

In the animal body a similar chain of reactions, in the reverse direction, has been assumed to occur during the easily performed oxidative conversion of propionate into carbohydrate (1).

A serious objection to lactate being an intermediate in the reduction of pyruvate in the propionic acid fermentation has, however, arisen. Chaix-Audemard (2) working in Fromageot's laboratory recently made the unexpected observation that pyruvate can be readily fermented in the presence of sufficient sodium fluoride to inhibit lactate decomposition completely. This observation strongly indicates that lactate is not a necessary intermediate between pyruvate and propionate. A similar situation exists in the four carbon monocarboxylic acid series. Lipmann

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and Perlmann (3) recently re-investigated the oxidation of butyrate to acetoacetate in animal tissues. In accord with earlier results of Friedmann and Maase (4) and Jowett and Quastel (5) they found that β-hydroxybutyrate is not an intermediate.

In the present communication the role of lactate in the reduction of pyruvate to propionate by the propionic acid bacteria will be re-examined.

Materials and Methods.

Dry preparations of *Propionibacterium pentosaceum*, strain E.2.1.4 from the collection of Prof. C. B. van Niel, were used in all experiments. Cultures were grown for 3–4 days at 30°C. in 3 liter flasks filled almost to the neck with the following medium: sodium lactate 15 g., yeast autolyzate 30 cc., tryptone 3 g., $M/1$ phosphate buffer pH 7.1 20 cc., $(NH_4)_2SO_4$ 1.5 g., $MgSO_4 \cdot 7H_20$ 0.05 g., distilled water to 1000 cc. The cells from 20–40 liters of medium were separated by centrifugation, washed once with distilled water, spread out in a thin (1–3 mm.) layer on watch glasses and dried rapidly *in vacuo* over P_2O_5 at room temperature*. A few grams of cells could be dried in 2–3 hours, but larger batches were left overnight. The dry bacteria were stored at 5°C. For use a weighed quantity was finely ground in a mortar and suspended in an appropriate buffer solution.

The dry bacteria were 25 to 40 per cent as active ($Q_{CO_2}^{N_2}$ = 9–20) as fresh cell suspensions ($Q_{CO_2}^{N_2}$ = 25–51) in the anaerobic decomposition of pyruvate. When stored at 5°C. they retain their full activity for at least several months. The rate of pyruvate decomposition increases with decreasing pH (Table I) over the range pH 5.3–8.0. A somewhat similar pH-rate relation was observed previously by Wood, *et al.* (17) using a suspension of living bacteria. In most of our experiments a pH 6.2 buffer was used. Besides pyruvate (Table I), the dried bacteria also ferment a variety of other substrates, including lactate, glucose, arabinose, glycerol, erythritol, and mannitol. In fact all compounds attacked by fresh cells are also decomposed by dried cells. The number of living bacteria in the dried preparations was not determined accurately, though

* This very simple and satisfactory technique for obtaining active and stable preparations of dry bacteria was developed by Lipmann (6) in his studies on pyruvic acid oxidation, by *Lactobacillus delbrückii*. The same technique was successfully applied by Duodoroff, *et al.* (7) in their work on the phosphorolysis of sucrose by *Pseudomonos saccharophila*.

it was observed that at least 10% of the cells in a two-day old preparation were viable. The dried cells differ from fresh bacteria in their ability to cause an accumulation of phosphate esters under suitable conditions. In this respect they are similar to the toluene-treated cells of Wood, Stone, and Werkman (8).

Most experiments were carried out by the usual Warburg manometric techniques. Lactate determinations were done by the colorimetric method of Barker and Summerson (9); when pyruvate was present in large amounts it was destroyed

TABLE I

Influence of pH on Pyruvate Decomposition

Initial pH of buffer	Relative rate
5.3	100
5.9	90
6.2	82
6.6	63
7.0	49
7.4	37
7.7	29

0.8 cc. of a 5 per cent suspension of dried bacteria in water + 0.1 cc. *M*/5 sodium pyruvate + 0.2 cc. *M*/2 phosphate buffer of the indicated pH. Gas phase: N_2. Incubation at 30°C. was continued until the pyruvate was all decomposed as judged by a sharp drop in the rate of CO_2 evolution. Relative rates were calculated from the reciprocals of the decomposition times.

by a preliminary treatment with H_2O_2, and excess peroxide was removed by evaporating the final aliquot to dryness on the steam bath. Pyruvate was estimated either by the bisulfite-binding method of Clift and Cook (10) or the more specific colorimetric method of Lu (11). Volatile acids were determined by steam distillation, followed by a Duclaux distillation. Pyruvate, when present, was removed by a preliminary treatment with 2, 4-dinitrophenylhydrazine in acid solution.

Of the chemicals used only acrylic acid requires special comment. Eastman Kodak Co. sodium acrylate was found to contain a large percentage of carbonate and possibly other non-volatile substances. Pure acrylic acid was separated from the commercial preparation by acid steam distillation. The product so obtained reacted with the theoretical amount of bromine.

Experimental

Influence of Fluoride on Lactate and Pyruvate Fermentations

Our experiments (Table II) confirm the observation of Chaix-Audemard (2) that lactate fermentation is much more sensitive to fluoride

than pyruvate fermentation. The rates of both processes are progressively inhibited by increasing concentrations of fluoride but, whereas, lactate decomposition (measured by either carbon dioxide production or lactate disappearance) is practically stopped by $M/160$ fluoride, pyruvate decomposition is only about 30 per cent inhibited at this concentration. Even with $M/10$ fluoride, pyruvate is still fermented at an appreciable rate. In most subsequent experiments $M/10$ or $M/40$ NaF was used when it was wished to eliminate lactate fermentation completely.

TABLE II

Fluoride Inhibition of Lactate and Pyruvate Fermentations

NaF conc.	Percentage inhibition Lactate	Pyruvate
0	0 (Q_{CO_2} = 5)	0 (Q_{CO_2} = 4)
$M/800$	26	
$M/400$	58	
$M/160$	96	29
$M/80$	100	57
$M/40$		62
$M/20$		79
$M/10$		87

1 cc. of a 5 per cent dried cell suspension containing $M/50$ phosphate pH 6.47. Substrate: 0.2 cc. $M/5$ sodium pyruvate or 0.2 cc. $M/1$ lithium lactate. NaF as indicated. Total volume 2 cc. Gas phase; N_2. Temperature: 30°C.

Reactions of Lactate in the Presence of Fluoride

The fermentation of lactic acid is essentially an oxido-reduction between molecules of lactic acid, or derivatives thereof, which form the oxidants and the reductants, respectively, of opposing O/R systems:

(1) $[\text{Lactic acid}]^{O} + [\text{Lactic acid}]^{R} = \text{Propionic acid} + H_2O + \text{Pyruvic acid}$

(2) $[\text{Lactic acid}]^{O} + [\text{Pyruvic acid} + H_2O]^{R} = \text{Propionic acid} + H_2O + \text{Acetic acid} + CO_2$

Grouped according to O/R systems we expect:

System O: Lactic acid + 2H $\rightleftarrows$ Propionic acid + H_2O

System R: Lactic acid $\rightleftarrows$ Pyruvic acid + 2H

The point of attack of fluoride may be on either one of these systems.

However, Chaix-Audemard has already shown that the aerobic oxidation of lactate at least is not influenced by fluoride. We confirmed this

observation and showed further that lactate is rapidly oxidized anaerobically in the presence of $M/10$ fluoride when fumarate or malate is provided as an oxidant. Pyruvate will also bring about the oxidation of lactate, though at a slower rate.

The rate of lactate oxidation by either O_2 or fumarate in $M/10$ fluoride is considerably more rapid than the rate of pyruvate fermentation. Therefore pyruvate accumulates as a product of lactate oxidation (Table III).

The ability of lactate and other compounds to be reduced was determined by using them as oxidants in a system containing glucose or

TABLE III

Oxidation of Lactate to Pyruvate in the Presence of Fluoride

Substrate	Gas Phase	Maximum rate	Lactate	Pyruvate
		cmm./hr.	*cmm.*	*cmm.*
None added.	N_2	30 (CO_2)		
Lactate...	N_2	30 (CO_2)	−20	48
Lactate + fumarate.	N_2	236 (CO_2)	−2,000	1,702*
Pyruvate	N_2	222 (CO_2)		
None added......	air	−127 (O_2)		
Lactate	air	−580 (O_2)	−1,620	1,567

* This value is corrected for the pyruvate formed from lactate alone.

Each vessel contained 3 cc. of a 5 per cent suspension of dried bacteria in $M/50$, pH 6.2 phosphate buffer containing $M/10$ NaF. 0.2 cc. $M/2$ lithium lactate, 0.2 cc. $M/2$ sodium pyruvate, or 0.3 cc. $M/2$ sodium fumarate was added as indicated. Total volume: 3.5 cc. Gas phase: N_2 or air. With air, the well contained 0.2 cc. KOH. Incubation: 90 minutes at 30°C. Samples were analyzed immediately after adding the substrate and at the end of the incubation period.

glycerol plus phosphate, fluoride, and bicarbonate. In the presence of an oxidant the glucose (or glycerol) is converted to phosphoglyceric acid which liberates carbon dioxide from the bicarbonate so the reaction can be followed manometrically. Without an oxidant little or no phosphoglyceric acid is formed.

We have found that pyruvate, malate, fumarate, oxaloacetate, and to a slight extent α-ketoglutarate can act as oxidants in the above system but lactate cannot do so. This shows that the reduction of lactate in system O, in contrast to its oxidation in system R, is inhibited by fluoride. Illustrative data are given in Table IV.

Fermentation of Pyruvate in the Presence of Fluoride

Since fluoride prevents the reduction of lactate, one might expect that the fermentation of pyruvate, normally yielding propionic acid, would be changed in the presence of fluoride to a simple dismutation giving lactic and acetic acids and carbon dioxide as end products. Such a result would be in accordance with the classical theory of the mechanism of the reduction of pyruvate to propionate. Our experiments have shown, however, that this does not occur. Lactic acid does not accumulate when pyruvate is fermented in the presence of sufficient fluoride to stop lactate fermentation. With or without fluoride, the main product of pyruvate

TABLE IV

Compounds Used as Oxidants in the Presence of NaF

Substrates		CO_2	Inorg. phosphate
		cmm.	*cmm.*
Expt. 1	None	80	(0)
	Glucose	100	14
	Glucose + pyruvate	542	−224
	Glucose + lactate	162	0
Expt. 2	Glycerol + ketoglutarate	272	−87
	Glycerol + oxalacetate	693	−412
	Glycerol + malate	545	−433
	Glycerol + fumarate	494	−398

1 cc. of a 5 per cent dried cell suspension containing $M/60$ phosphate, $M/1$ NaF, and $M/10$ $NaHCO_3$. 0.2 cc. $M/4$ substrates added as indicated. Total volume: 2 cc. Gas phase: CO_2. Temperature: 30°C.

reduction is propionic acid (Table V). In a number of other experiments with pyruvate alone or with glucose plus pyruvate very little if any lactate was formed (Table VI). These results lead unavoidably to the conclusion that lactic acid is not an intermediate in propionic acid formation under these conditions.

Other Possible Intermediates between Pyruvate and Propionate

The elimination of lactate as an intermediate in the reduction of pyruvate to propionate, made it desirable to look for other possible intermediates. So far the results of this search have been entirely negative. Acrylate, thought by some investigators (12, 16) to be a possible precursor of propionic acid, is not fermented by our preparations either

in the presence or absence of fluoride; also it cannot act as an oxidant for glucose or as a reductant for fumarate. Other compounds not attacked at an appreciable rate are alanine, cysteine, phosphoserine. Glyceric acid is fermented in the absence of fluoride, but much too slowly to function as an intermediate. Phosphoglyceric acid is also eliminated because it is not decomposed in the presence of fluoride.

TABLE V

Products of Pyruvate Decomposition With and Without NaF

Product	*mM*/100 *mM* fermented pyruvate: No NaF	*M*/40 NaF
Propionic acid	31	52
Acetic acid	54	47
Carbon dioxide	60	73

8 cc. of a 5 per cent suspension of dried bacteria containing *M*/60 phosphate buffer, pH 6.24, and 1 cc. of *M*/1 sodium pyruvate. Gas phase: N_2. Incubated 210 minutes (no NaF) and 480 minutes (with NaF) at 30°C. Data are corrected for a control without substrate.

TABLE VI

Lactate Production from Pyruvate in the presence of NaF

Substrate	Carbon dioxide	Lactate formed
	cmm.	*cmm.*
Pyruvate	558	<10
Pyruvate + glucose	823	$\lesseqgtr$25

1 cc. of a 5 per cent suspension of dried bacteria containing *M*/60 phosphate, *M*/10 $NaHCO_3$, and *M*/18 NaF. 0.1 cc. *M*/5 glucose or *M*/2 sodium pyruvate added as indicated. Total volume: 1.2 cc. Gas phase: CO_2. Incubated 205 minutes at 30°C.

Discussion

For a step by step interpretation of the propionic acid fermentation two of our results seem of major importance. (1) The reduction of lactate may be completely blocked without affecting pyruvate fermentation severely. (2) With lactate reduction blocked, little or no lactate accumulates when pyruvate is decomposed either dismutatively or by grouping it with glucose or glycerol. The first result shows that pyruvate *may* be reduced without passing lactate. The second result makes it unlikely that lactate is even on the main pathway of the normal, uninhibited reduction of pyruvate to propionate.

In view of these results the following scheme is proposed for the conversion of pyruvate and lactate to propionate:

$$\begin{tikzcd}
\text{Pyruvate} \arrow[r, "+2\text{H}"] \arrow[dr, shift right, "+2\text{H}"'] & X \arrow[r, "+2\text{H}"] & \text{Propionate} \\
& \text{Lactate} \arrow[ul, shift right, "-2\text{H}"'] \arrow[u, "-\text{NaF}"'] &
\end{tikzcd}$$

The two pairs of hydrogen atoms required for the reduction of pyruvate to propionate are of course supplied by dehydrogenation of carbohydrate, pyruvate, lactate, or other fermentable substrate. Because lactate is so readily fermented, it is assumed to be converted to the true intermediate (X) by means of a fluoride-sensitive side reaction. Some lactate may also be converted to propionate via pyruvate but this roundabout pathway is too slow to account for the normal rate of lactate decomposition.

The nature of the hypothetical compound X is at present entirely obscure. A transformation of lactate most easily fitting into the general theory of biological oxidations would be a dehydration to acrylate:

$$CH_3 \cdot CH(OH) \cdot COOH \rightarrow CH_2{:}CH \cdot COOH + H_2O$$

Such a reaction, however, is not supported experimentally. Acrylate is entirely inert as a substrate for the propionic acid bacteria. It may be noted, however, that acrylate seems to be attacked by liver tissue which, as previously mentioned, converts propionate into carbohydrate by an oxidative process (4).

Although the details of the mechanism of propionate formation are not clear it is evident that a close analogy exists between the reduction of pyruvate which does not involve lactate and the oxidation of butyrate which does not go over β-hydroxybutyrate. We believe that a chain of reactions between keto and fatty acids which does not involve a hydroxy acid will prove of rather general biochemical importance, applying not only to propionic and butyric fermentations and oxidations but also partially to the succinic and citric acid cycles; cf. also (3) and (14). In this connection it may be mentioned that fluoride inhibits rather specifically the oxidation of butyric acid in liver (5). A connection between the fluoride effect on lactate fermentation by *P. pentosaceum* and that on carbon dioxide fixation and succinate formation observed by Wood and Werkman (13) with the same organism is possible but not too likely.

The observation that the oxidation of lactate to pyruvate is much faster than the reverse reaction pyruvate to lactate is somewhat surprising and may be correlated with the nature of the enzyme systems catalyzing these reactions. In this connection it is significant that the accumulation of pyruvate does not inhibit the oxidation of lactate although keto acids very generally do inhibit hydroxy acid oxidations (15). Most of these reactions are catalyzed by pyridine enzymes, and the inhibition of keto acid is due at least partly to the fact that the potential of the pyridine catalyst is lower than that of the hydroxy-keto-acid system. Consequently an accumulation of keto acid strongly favors the reverse reaction, *i.e.*, the formation of hydroxy acid. The ease of oxidation of lactate by propionic acid bacteria and its insensitiveness to an accumulation of the oxidation products suggests the participation of a different type of lactic dehydrogenase possessing a normal potential higher than that of the pyridine coenzymes, analogous to the yeast lactic dehydrogenase of Bach, *et al.* (18).

Summary

The fermentation of lactate by dried preparations of *P. pentosaceum* is much more sensitive to fluoride than is the fermentation of pyruvate. Fluoride inhibits the reduction but not the oxidation of lactate. In the presence of sufficient fluoride to prevent lactate reduction entirely, pyruvic acid is still reduced to propionic acid. This indicates that lactate is not an intermediate in pyruvate reduction under these conditions and probably not even in the absence of fluoride. Acrylate, alanine, cysteine, phosphoserine, glyceric acid, and phosphoglyceric acid have been excluded as possible intermediates in pyruvate reduction. Evidence is presented which indicates that the oxidation of lactate by *P. pentosaceum* is not catalyzed through the usual pyridine coenzymes.

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An Electrophoretic Study of Gliadin[1]

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Introduction

Although the researches of T. B. Osborne on the vegetable proteins led to the conclusion that gliadin, the fraction of wheat gluten which is soluble in 60–70% alcohol, was a single protein, the results of more recent studies have indicated that gliadin is a complex consisting of at least two interacting components. Several groups of workers, using fractional precipitation or peptization methods, have found that gliadin can be separated into various fractions which show progressively changing properties. Haugaard and Johnson (5) found that gliadin could be fractionated by chilling its solution in alcohol first to 0°C. and then to −11°C. This fractionation was completely reversible, and Sørenson (21) concluded that gliadin constitutes a "reversible dissociable component system." Sandstedt and Blish (17) and McCalla and Rose (16) reached similar conclusions with regard to the whole gluten.

Studies involving physical measurements on gliadin (6, 9, 3) have failed to arrive at any consistent and clear cut picture as to its nature further than the recognition that it consists of a mixture of components which show a marked tendency toward interaction among themselves. No definite knowledge has been gained as to the number or relative amounts of these components or as to their manner of interaction.

Electrophoresis techniques have proved to be of value in the detection and study of complex formation and interaction occurring between components of mixtures containing proteins. While the most complete study in this respect is that of Longsworth and MacInnes (14) on mixtures

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of ovalbumin and yeast nucleic acid, there are numerous instances where electrophoresis studies have led to the interpretation that interaction occurs between one protein and another (12, 4, 19, 7, 8) or between a protein and some non-protein component (22, 18, 15) under certain conditions in the solutions of the mixtures.

In mixtures of proteins or of proteins and other colloid electrolytes where no interaction of these components occur, the components migrate independently in an electric field. Patterns for the ascending and descending boundaries are symmetrical with respect to number and relative areas under the peaks, and the mobility of each component is not affected by the presence of the other. In mixtures where interaction between components does occur, the electrophoresis patterns are not symmetrical as to number and/or the relative areas under the peaks, and the mobilities of one or more peaks in each leg will vary from that which is characteristic of any component alone. If a protein preparation of unknown composition develops a series of peaks which are symmetrical with respect to relative area, number, and relative mobility in the two legs, the preparation is recognized as a mixture of non-interacting components. Where a protein preparation develops marked asymmetries in the electrophoresis patterns for the two legs, the preparation must be assumed to be a mixture of interacting components.

In protein systems which consist of mixtures of components and in which interaction occurs between the components, we may assume that the forces giving rise to the interaction may belong to two distinct types. The association may arise from electrical interaction of the molecules involving ionized groups of opposite charge, or the interaction may be more specific in character and involve hydrogen-bonding or similar forces and be dependent, insofar as intensity is concerned, upon specific spatial configurations along the surfaces of the interacting molecules.

While it is recognized that electrical (ionic) interaction will occur most readily in *protein* mixtures when the pH of the medium is such as to give one component a net positive and the other a net negative charge, it is not possible to foretell just how far the pH of the system must be removed from the interisoelectric region of such amphoteres before ionic interaction will become negligible. It is conceivable that, if the mosaic of charged groups on the surfaces of two amphoteric molecules approach a mirror image arrangement with respect to sign and spatial distribution, ionic interaction could persist into pH ranges relatively far removed from the interisoelectric range. The more random the distribution of the

positively and negatively charged groups existing on the surfaces involved, the less would be the probability of complex formations beyond the interisoelectric pH range. On the other hand, if one component of a mixture were protein (amphoteric) while the other were actually or potentially a relatively low molecular weight electrolyte (such as a detergent), ionic interaction could occur in ranges of pH where both components possessed the same sign of net charge provided there still existed ionized groups, on the protein, having a sign of charge opposite to that of the net charge and which would form relatively non-ionizable salts with the heavy ion of the other component.

It is not possible, therefore, to assume, in those cases where complex formation is observed in pH ranges relatively far from the interisoelectric range of the components, that the forces causing complex formation are not ionic in character. However, since Longsworth and MacInnes (14) in their analysis of the system, ovalbumin+nucleic acid, found interaction between these components to persist for only a very short range of pH above the isoelectric point of the protein, it would appear that ionic interaction might, in general, be expected to become insignificant beyond the range in which the net charges on the components were of opposite sign. It seems especially probable that, in *protein* systems showing evidences of interaction of components in pH ranges more than a short distance from the interisoelectric range, the interacting forces are not wholly ionic in character.

Where electrical (interionic) forces have been recognized as the forces giving rise to interaction in mixed systems containing protein, the electrophoresis patterns obtained have indicated (14, 12, 4) that this type of interaction is weakened at higher ionic strengths with the result that the patterns become more symmetrical. This tendency toward suppression of interaction at high ionic strengths thus may be used to determine whether the interaction is or is not ionic in character in any given case. If the type of interaction involved is ionic, the patterns should become more symmetrical with increasing ionic strength. If the type of interaction is not ionic, it is still possible that changes in ionic strength may modify the patterns. In such a case, however, it might be expected that the change caused by the electrolyte (a) would be specific in character, *i.e.*, would vary with the nature of the buffer at constant ionic strength and pH, and (b) the change toward more symmetrical patterns in the two legs of the cell would not necessarily become more pronounced as ionic strength increased.

Experimental

Early results from the electrophoretic analysis of gliadin indicated not only that this protein is electrophoretically heterogeneous but also that the components of which it consists do not migrate as independent entities in the electrical field. At all pH ranges where it is possible to obtain the protein in solution, evidences of interaction of components were observed.

A primary difficulty in working with gliadin is its limited solubility. A series of preliminary experiments were carried out in order to determine the most satisfactory condition for the electrophoretic study of the protein. Various kinds of buffers were used at pH values between 2.4 and 10.3 and at ionic strengths between 0.002 and 0.20. Since gliadin is only slightly soluble in the region of its isoelectric point, and since its solubility in all ranges of pH is markedly decreased by the presence of higher concentrations of salts, the range in which satisfactory concentrations of the protein may be obtained is limited. These solubility experiments with various buffers and at varying ionic strength led to the conclusion that optimal solubility of gliadin through the greatest range of ionic strength was attainable in acetate buffers of pH 3.8–4.0. This pH and buffer system was chosen as standard and all electrophoresis experiments reported in this paper were run with acetate buffers of pH $= 3.8 \pm 0.1$.

The Tiselius electrophoresis apparatus and the techniques employed were, in general, those described by Longsworth (11).[4]

Four different preparations of gliadin were used in these experiments. Gliadins I[5] and III were prepared by the alcohol method of Osborne, and gliadins II and IV were prepared by the acetic acid peptization method of Blish and Sandstedt (1). Each of these preparations was precipitated at least four times. Gliadins I, II, and III were prepared from commercial mixed flours while gliadin IV was made from a pure durum wheat flour. With the exception of gliadin II, which had a light tan color, all of the powdered preparations were completely white. Since glia-

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[4] The curves presented in Figs. 1–4, inclusive, are the dn/dx patterns (n = refractive index, x = distance along the leg of the cell) for the ascending (asc) and descending (dec) boundaries in the electrophoresis cell after the time-field strength interval designated. These curves represent tracings of the projected scanned patterns. In the figures the initial boundary is represented by the vertical line at the tail of the arrow used to designate the direction of migration. A vertical mark on the top of the arrow is made at a distance from the starting point which is equivalent to a distance of 1 cm. in the electrophoresis cell. At pH 3.8, the protein carries a positive charge so that the direction of the arrow shown in all the figures is toward the cathode.

[5] We are indebted to W. E. Westlake for this preparation of gliadin.

din I gave the clearest solutions, it was used in most of the electrophoresis experiments.

The approximately one per cent gliadin solutions used for electrophoresis were made up by allowing as much as was soluble of 0.5 g. of gliadin to dissolve in 50 cc. of buffer. After solution had been allowed to proceed for 48–96 hours at 6°C., the undissolved residue was centrifuged out and the solutions were then dialyzed for at least 40 hours against two liters of the buffer at 4–6°C. Higher and lower concentrations of gliadin were prepared in a similar fashion using more or less gliadin as the case might be. Estimates of the amounts of gliadin existing in solution in the various experiments were obtained by comparing the areas under the curves with the area obtained with a standard solution for which the protein concentration was determined by nitrogen content.

Fig. 1 shows the electrophoresis patterns obtained at pH 3.8 and at ionic strengths between 0.002 and 0.10 on the gliadin which dissolved in 50 cc. of each buffer when equilibrated with 0.5 g. of the protein. The amount of protein which dissolved was nearly quantitative for ionic strengths below 0.02 but decreased sharply above that ionic strength. The actual amounts of protein present in each solution are given in the legend to Fig. 1 along with the field strength and the time of electrophoresis.

It may be seen from the patterns in Fig. 1 that evidences of complex formation between components (pattern asymmetry) are present at all ionic strengths and that no progressive decrease in this tendency is observed as the ionic strength is increased. In the systems of ionic strength around 0.01 the diagrams indicate the separation of a slow fraction which lags behind in the descending leg and of a fast fraction which moves out in front in the ascending leg of the cell. This phenomenon is suppressed and finally disappears at higher ionic strengths. The tendency for the fast fraction to separate is also entirely absent at ionic strengths lower than 0.01, although the area under the peak corresponding to the slow fraction increases at lower ionic strengths. It must be emphasized that the amount of protein which goes into solution at ionic strengths above 0.02 is less than quantitative, and the variations in the shapes of the diagrams at these higher ionic strengths may in part be due to actual differences in proportions of the components of the whole gliadin, resulting from selective solubility. This possibility has not yet been thoroughly investigated.

A peculiar phenomenon observed in gliadin solutions at ionic strengths above 0.06 is illustrated in Fig. 1, Diagrams 5 and 6. If the field strength at which the solution is allowed to undergo electrophoresis is not greater than 4–5 volts per cm., the patterns obtained are smooth curves. If the field strength is somewhat higher (*i.e.*, 7–8 volts/cm.) and the field

strength-time product is held constant, the general contour of the patterns remains similar to those obtained at lower field strengths but a marked tendency to peak up in all regions of the patterns is shown. The obvious explanation that these peaks might be due to heating in the

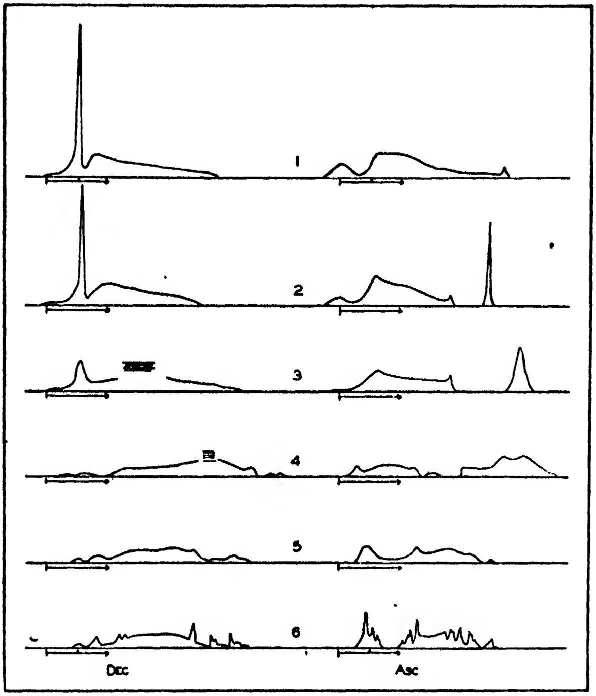

FIG. 1

Electrophoresis Patterns for Gliadin at pH 3.8 in Acetate Buffers of Varying Ionic Strengths

(1) 0.95% gliadin, 0.002 ionic strength, 7600 seconds at 7.00 volts/cm.
(2) 0.95% gliadin, 0.01 ionic strength, 8140 seconds at 7.25 volts/cm.
(3) 0.80% gliadin, 0.02 ionic strength, 21,640 seconds at 5.12 volts/cm.
(4) 0.73% gliadin, 0.06 ionic strength, 25,700 seconds at 7.25 volts/cm.
(5) 0.64% gliadin, 0.10 ionic strength, 57,000 seconds at 4.12 volts/cm.
(6) 0.60% gliadin, 0.10 ionic strength, 32,600 seconds at 7.25 volts/cm.

cell, with convection currents giving rise to the peaking, seems to be disproved by the fact that solutions of monophoretic gums and proteins (gum arabic or β-lactoglobulin) as well as an inert material (sucrose) under similar conditions of ionic strength and field strength give no such

peaking and by the fact that, if the gliadin is subjected to identical conditions as to concentration of protein, field strength, ionic strength, and type of buffer, etc., the peaked diagrams can be repeated on separate runs within a very small degree of variation as to number, position, and area under the peaks obtained. While an explanation of this phenomenon can not yet be given, it seems possible that, if complexes involving varying ratios of the components exist in the gliadin solution, a higher field strength might effect a greater relative separation than a low field strength due to the fact that the distance a given complex could move during its "life time" would be greater at high than at low field strengths. Each peak then would not necessarily indicate one complex but the peaking would nevertheless be the result of the existence of complexes in the system.

From the patterns obtained for the ionic strength series, it appeared that an ionic strength of 0.01 showed the greatest promise with regard to separation of the components. Consequently our further electrophoretic studies of gliadin have been conducted under these conditions, *i.e.*, at ionic strength = 0.01, and pH = 3.8 in acetate buffers (and at a temperature of 0.5°C.).

In order to determine the effect of the source and method of preparation of gliadin on its electrophoretic pattern, runs were made on each of our four preparations of gliadin. The concentration of gliadin in each case was about 0.95 per cent. The field strengths used in these runs were between 7.24 and 8.20 volts/cm. but all runs were continued to a field strength-time product of 58,900 volt-seconds/cm. The patterns obtained in this series of runs are shown in Fig. 2. Identical runs made with any gliadin preparation gave patterns which were exactly superimposable.

The similarity of these curves offers confirmation of the observation of Blish and Sandstedt (1) that gliadin prepared by the acetic acid method is identical with that prepared by the older alcohol method of Osborne. The differences between these patterns, on the other hand, while relatively small in magnitude, must be taken to indicate that every preparation of gliadin is likely to show small differences in composition or ratio of components. It is of interest that there is no significant difference between a gliadin prepared from a pure durum flour and those preparations which were made from a commercial mixed flour.

A series of experiments using a single preparation of gliadin (gliadin I) under the standard conditions of pH, ionic strength, and temperature,

demonstrated the following facts. (a) If the protein concentration and the field strength were held constant, the patterns obtained, at the end of

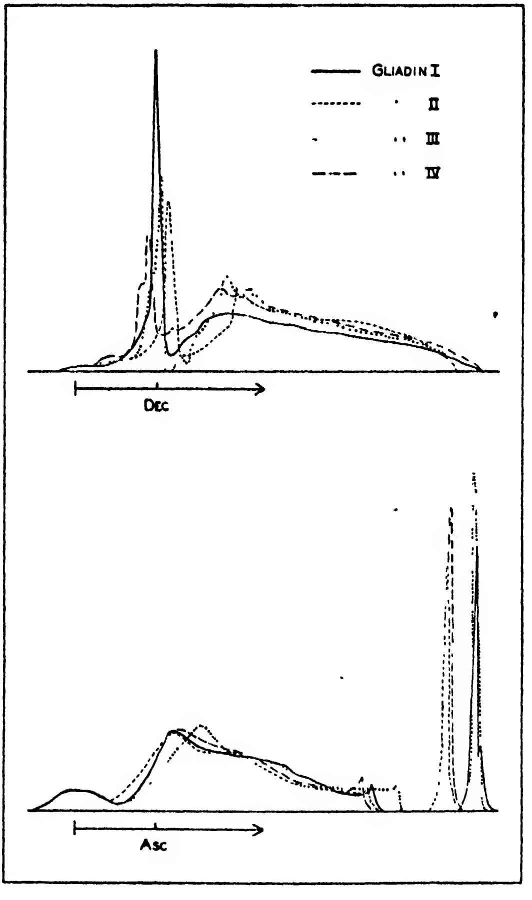

FIG. 2

Electrophoresis Patterns for Four Different Preparations of Gliadin at 0.01 Ionic Strength in Acetate Buffer of pH 3.8 and Gliadin Concentration of 0.95% Samples I and IV prepared by alcohol method and samples II and III prepared by acetic acid method. Patterns taken at 58,900 seconds volt/cm.

equal time intervals of electrophoresis, were identical in every detail. (b) If the protein concentration and field strength were held constant the *mobility* of every recognizable peak in the patterns was constant

and did not vary with time of electrophoresis. This is in contrast to the observation of Longsworth and MacInnes (14) to the effect that the mobility of the complex in mixtures of egg albumin and nucleic acid varied progressively with time of electrophoresis. (c) If protein concentration was held constant, the mobility of the fractions corresponding to each recognizable peak in the patterns was independent of the field strength. Three experiments made with 0.95 per cent gliadin I at field strengths of 3.63 v./cm., 7.54 v./cm., and 13.52 v./cm., respectively, but all run for a total of 60,000 volt-sec./cm. gave patterns which were superimposable. Peaks were progressively sharper as the field strength was increased but areas under the peaks and positions of the peaks were exactly the same for all three. This is added confirmation that the mobility of no fraction of the gliadin varied with time of electrophoresis and, by itself, might be taken to indicate that every fraction of the gliadin was actually migrating independently in solution. (d) If the field strength was held constant and the protein concentration was varied, the general appearances of the patterns obtained were somewhat similar but the *mobilities* of the peaks and the areas under these peaks varied progressively with change in protein concentration.

This effect of the concentration of gliadin upon the nature of the patterns is demonstrated by the following experiment. As much as was soluble of 2.0 g. of gliadin I was dissolved in 100 cc. of the standard acetate buffer. After removal of the small amount of insoluble material, a fraction of this solution was diluted with an equal volume of buffer and another fraction diluted with three volumes of buffer. Each of these solutions was placed in a dialyzing sac and all three were placed in the same 2 liters of the buffer and allowed to equilibrate at 6°C. for 48 hours. The electrophoresis pattern for each was then made at a standard field strength of 7.20 v./cm., and the patterns were scanned at the end of 8140 seconds. The patterns so obtained are shown in Fig. 3. The initial protein concentrations were found to be 1.84 per cent, 0.92 per cent, and 0.46 per cent for the three solutions. At all concentrations, the separation of a fast fraction in the ascending leg and of a slow fraction in the descending leg are in evidence, but an examination of these patterns indicates that the fast fraction separating from the solution of higher protein concentration moves with a higher mobility than the corresponding fraction separating from the lower concentrations of protein. This fraction constitutes, too, a lower percentage of the total protein when separating from the higher protein concentration. Converse relationships

apply to the mobilities of the slow fractions separating in the descending leg. In Table I are given the mobilities of the fast and slow fractions to-

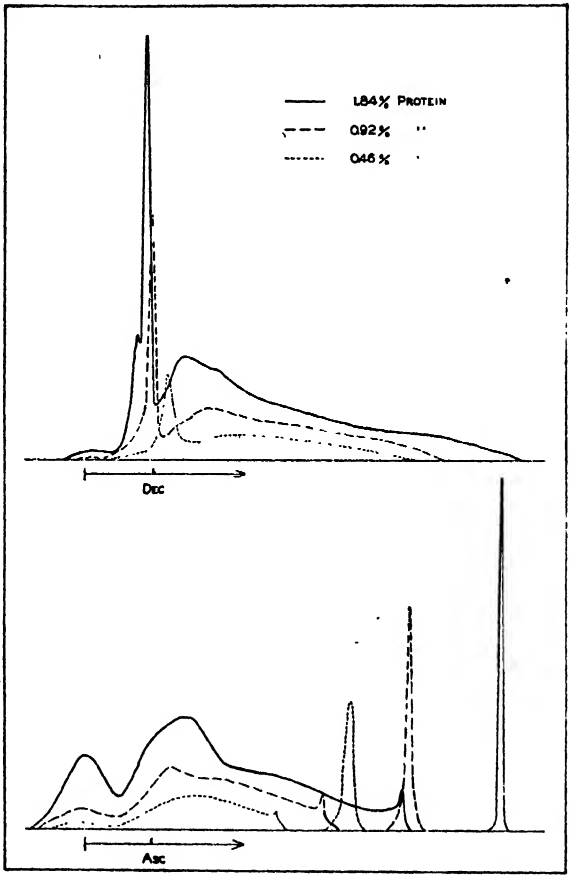

Fig. 3

Electrophoresis Patterns of Gliadin I at 0.01 Ionic Strength in Acetate Buffer of pH 3.8 for Three Concentrations of Gliadin
Patterns all taken at 58,600 seconds volt/cm.

gether with the ratios of the amount of protein corresponding to the fast peak (F) and the slow peak (S) to the total protein (T) as obtained from the areas under the curves.

Since the fast and slow fractions which separate from gliadin at dif-

ferent initial concentrations constitute variable percentages of the total protein and, especially, since these separated fractions show different mobilities, it must be concluded that they cannot be regarded as pure components but rather as mixtures of components in variable proportions dependent upon the original concentration of the protein from which they separate.

A preliminary attempt was made to isolate these fractions, using a 2.5 per cent gliadin solution as the parent material. (This high concentration was used because the purity of the fast and slow fractions, as indicated by mobilities, would be expected to be greatest when separated from a protein solution of high initial concentration.) Since the separation cells designed by Tiselius were not available, use was made of a simple U-tube having a stopcock and capillary at the bottom of the U through

TABLE I

Effect of Concentration of Total Protein on the Mobilities and Relative Amounts of Fast and Slow Fractions of Gliadin as Obtained by Electrophoresis in Acetate Buffer; pH = 3.8 and μ = 0.01

Protein Conc.	Mobility cm²/v. sec. × 10^{-4}		Relative areas	
%	F	S	(F)/(T)	(S)/(T)
1.84	0.96	0.13^7	0.075	0.145
0.92	0.75	0.15^6	0.138	0.227
0.46	0.61	0.19^0	0.257	0.279

which the protein solution could be introduced. The U-tube was suspended from the side-arms of the electrode vessels by the rubber sleeves used with the regular Tiselius cells. The fractions were removed by means of a capillary pipette, using the compensator syringe for suction, as suggested by Longsworth (11). The separation was carried out in the 0.01 *N* acetate buffer of pH 3.8 as used for the previous runs. To prevent depletion of the buffer during the rather long run necessary to effect separation, a layer of 0.1 *N* buffer was introduced into each electrode vessel above the 1 *N* potassium chloride solution which surrounded the electrodes. This precaution is due to Tiselius (23). Since the U-tube was constructed of 19 mm. tubing, the portion of the tubing included within the slits of the mask covering the schlieren lens had a sufficiently small curvature so that the separation process could be observed by the Philpot-Svensson method with only a small amount of distortion. After 60,000 seconds at an applied potential of 255 V. about 30 cc. of solution containing the fastest moving component could be withdrawn and,

following a similar run of 75,000 seconds, about 15 cc. of solution representing the slow fraction was obtainable.

The solutions withdrawn in this way were examined separately by making electrophoretic runs upon each of them in the standard (acetate) buffer (μ = 0.01, pH 3.8). The patterns obtained in these runs are shown

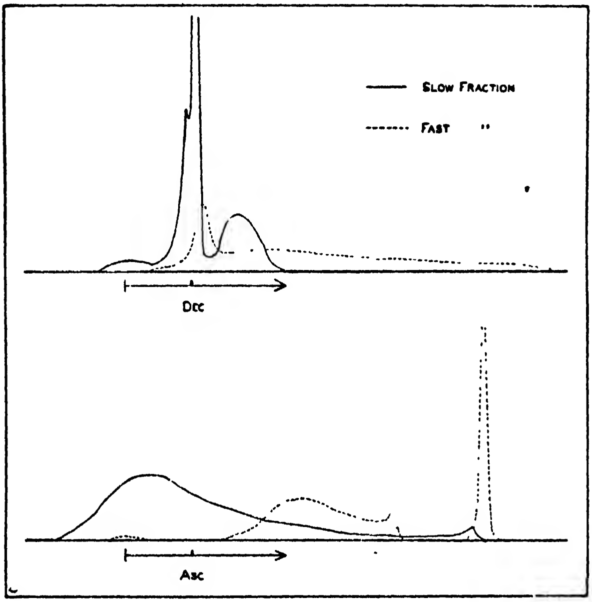

FIG. 4

Electrophoresis Patterns for Slow Fraction (at 0.98%) and Fast Fraction (at 0.63%) of Gliadin Obtained by Electrophoretic Separation of Whole Gliadin (2.5% Protein, 0.01 Ionic Strength, pH 3.8) and Re-run at 0.01 Ionic Strength in Acetate Buffer of pH 3.8
Slow fraction run 13,670 seconds at 7.26 volts/cm.
Fast fraction run 8200 seconds at 7.19 volts/cm.

in Fig. 4. It is evident from these patterns that these fractions are still mixtures. It seems unlikely that this is due entirely to contamination during removal of the fractions. The slow fraction appears to be less contaminated with faster components than the fast fraction is with slow components. Comparisons of these patterns with those of whole gliadin at exactly the same protein concentrations were not made but a rough comparison with the patterns of the concentrations series of Fig. 3 can

be made. It is obvious that the F/T ratio for the "slow" fraction is lower than for whole gliadin while the S/T ratio is higher. Similarly the F/T ratio for the "fast" fraction is higher than for whole gliadin while the S/T ratio is lower. A partial fractionation of the components of whole gliadin was certainly attained. Since only small quantities of these frac-

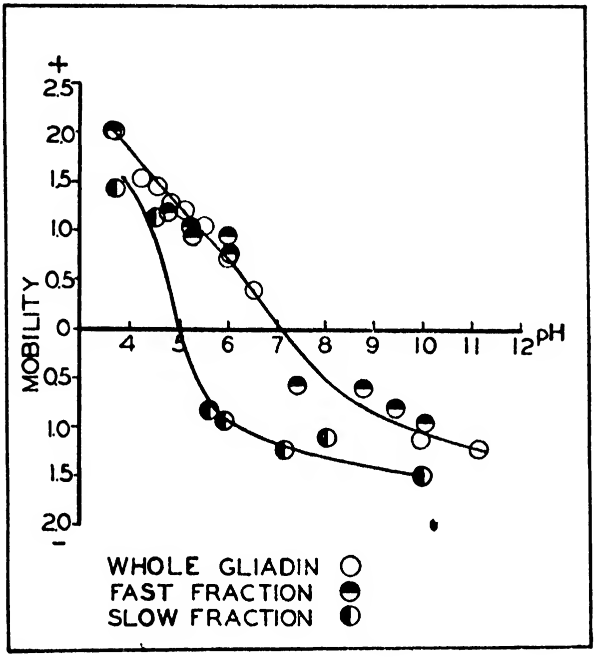

Fig. 5

Mobility-pH Curves Obtained for Slow and Fast Gliadin Fractions Obtained by Electrophoretic Separation of Whole Gliadin

Values given are those obtained by microelectrophoresis method with protein adsorbed on quartz particles in electrolyte solutions of constant acetate concentration (.0093 N in total acetate).

tions were obtainable, further study by the Tiselius method was impractical. For this reason, use was made of the microelectrophoresis cell (2) for the determinations of the isoelectric points of these fractions and of whole gliadin adsorbed on quartz particles.

Because of the probable specific interaction between gliadin and acetic acid, as evidenced by the peptization of gliadin by acetic acid in the Blish

and Sandstedt preparation of gliadin and by the work of Sinclair and Gortner (20), the runs made in the microelectrophoresis cell were made at a constant level of total acetate instead of at constant ionic strength. This was carried out by adding a constant volume of acetic acid to 1 cc. of the protein solution, adding sodium hydroxide until the desired pH was approached, and then diluting to a constant volume. This unorthodox procedure should serve to equalize any specific effect of the acetate system upon the mobility of the gliadin fractions but it also results in greatly decreased mobilities at higher pH values due to the concomitant increase in ionic strength. The level of total acetate used was one-tenth that in the standard 0.01 *N*, pH 3.8 buffer. Higher concentrations of total acetate enabled such a high amperage to be carried through the cell that thermal currents greatly disturbed the paths of the particles. A large number of particles were timed for each direction of migration in the cell at each pH. The results of these determinations are plotted in Fig. 5.

These mobility-pH curves (constant acetate concentration) indicate that the surfaces of quartz particles coated with whole gliadin or with the fast fraction have an identical composition. Presumably this is due to a preferential adsorption of the fast fraction on quartz. The isoelectric point of the fast fraction and of the fraction adsorbed at the quartz interface from whole gliadin was found to be at a pH of 7.0. The isoelectric point for the slow fraction was indicated to be at a pH of 5.0. This is probably a higher pH than would be characteristic of the pure slow component, especially in view of the apparent preferential adsorption of the fast fraction by quartz. The latter fraction, even if present in very small amounts relative to the slow fraction, would have a disproportionate effect in maintaining the apparent isoelectric point of this fraction at a high pH.

Discussion

While it is not possible to derive a dependable picture as to the exact physical constitution of gliadin on the basis of the electrophoresis data thus far obtained, there are certain conclusions which seem sufficiently substantiated to merit discussion. It is evident from these studies that gliadin is not an electrophoretically homogeneous material. It is equally apparent that the components of which it is constituted do not migrate independently in solution under any of the conditions so far studied. The occurrence of complex formation between the components is in-

dicated under all conditions by the pronounced asymmetry of the patterns obtained for the ascending and the descending boundaries and by the variation in mobilities and relative areas of the several fractions which may be separated under appropriate conditions.

The nature of this complex formation is problematic. Electrical interaction (interionic) appears not to account for the complex formation since increase in ionic strength of the systems fails to cause any noticeable decrease in the asymmetry or complexity of the patterns. Variation in ionic strength does, however, profoundly change the nature of the patterns.

Although conditions were found at which there was obtained a distinct separation of fractions from the main body of the complex, the fractions so obtained must still be regarded as complexes themselves, containing only a higher percentage of a fast or a slow component than is characteristic of the whole gliadin. This changed ratio of components in these fractions, as compared to the ratio characteristic of the original gliadin, is indicated both by electrophoretic patterns obtained on them and by the different isoelectric point values shown by them.

It is not yet possible to explain why for a given initial protein concentration, each peak in the patterns shows a constant mobility with time of electrophoresis or with varied field strength, while a change in the initial concentration of the protein results in a marked change both in the mobilities of the apparently corresponding peaks and a change in the proportion of total protein which will appear in the fractions separating in the region identified with these peaks.

It is necessary to conclude that the slow and fast fractions, while possessing a constant mobility, are still mixtures of components. Whether these represent varying proportions of only two primary components, or whether a greater number of primary components are present in the whole gliadin has not yet been determined. By repeated electrophoretic separation of these fast and slow fractions, and through electrophoretic analysis of fractions obtained by thermal or other methods of fractionation of whole gliadin, it is hoped that a final pure component representing each can be obtained. If this can be accomplished, then it should be possible, by recombining these components in varying ratios, to determine whether or not they represent the only components in gliadin. Something more could then be learned about the constants involved in the complex formation and the nature of the forces of interaction responsible for the existence of gliadin as such.

Summary

Gliadin is not an electrophoretically homogeneous protein. Tiselius patterns, obtained under a wide variety of conditions as to pH, kind of buffer, ionic strength, and protein concentration, indicate that the components of gliadin do not migrate as independent entities under any of these conditions, *i.e.*, there are definite evidences of component interaction under all conditions where it is possible to obtain the protein in solution. It is conclused that the interaction between components of this protein is not ionic interaction, or at least not completely so.

Various preparations of gliadin, when prepared by the same method or by different methods, give rise to electrophoresis patterns which are nearly identical. The small differences observed are taken to indicate, however, that small variations in ratios of components will exist in various gliadin preparations.

Mobilities of recognizable peaks in the gliadin patterns are not variable with time of electrophoresis nor with field strength. Changes in ionic strength, protein concentration, nature of the buffer system and pH cause radical changes in the contour of the patterns obtained.

Separation of fast and slow fractions from the main body of complex occurs in acetate buffers at pH 3.8 and at an ionic strength close to 0.01. These fractions are recognized to be mixtures of components. Isolation of these fractions in sufficient amounts to make it possible to further separate them into electrophoretically homogeneous components has not yet been attained. Isoelectric points determined on samples of these fractions, however, show that the isoelectric point of the fast fraction is at pH 7 while that of the slow fraction is at pH 5.

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Vitamin Synthesis by Torula Yeast

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Considerable quantities of feed yeast of the type known as "torula" yeast are produced commercially using wood sugar, sulfite waste liquor, and molasses as the sources of carbohydrate. Fruit wastes may also be utilized for the production of torula yeast. Nolte, von Loesecke, and Pulley (1) grew torula yeast (*Torulopsis utilis*) on citrus-waste press juice. In this Laboratory, Stubbs, Noble, and Lewis (2) have used a variety of fruit juices for obtaining high yields of torula yeast.

Torula yeast is of particular nutritional interest because of its high content of protein and B-complex vitamins. The protein content is fairly uniform, N $\times$ 6.25 averaging over 50 per cent of the dry yeast substance in most cases. The content of various factors of the vitamin B complex is more variable. The vitamin relations of *Torulopsis utilis* are of particular interest, since this yeast is autotrophic with respect to vitamins (3, 4) and possesses well-developed vitamin-synthesizing abilities. This report deals with vitamin synthesis by torula yeast grown on fruit-juice substrates.

Methods

Yeast Propagation. Torulopsis utilis was grown in fruit juice media in laboratory-scale copper fermenters with about 10 liters of working capacity. High conversions of sugar to yeast substance, in most cases averaging around 55 g. of dry yeast per 100 g. of sugar, were obtained by the use of adequate aeration and the incremental addition of nutrients.

Air was supplied at the rate of about 1.5 liters per minute per liter of medium. The sugar concentration was generally maintained around 0.5 per cent. The propagation period was usually 6 to 8 hours, during which a 10-fold increase in yeast, as

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* This is one of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

compared with the inoculum, was usually obtained. The temperature was maintained at 30° C. The pH was held between 4 and 5 and the nitrogen requirement supplied by the addition of ammonia. It was found that the amount of ammonia needed for pH control was in most cases approximately the same as the amount required for growth. (Torula yeast contains about 9 per cent of nitrogen.) After propagation the cultures were held overnight in glass bottles at 2° C., and then separated in the Sharples Super Centrifuge. Complete details of the propagation work are to be published elsewhere (2).

Vitamin Assays. Thiamin was determined by the thiochrome method of Conner and Straub (5). Riboflavin and pantothenic acid were determined by the *Lactobacillus casei* methods of Snell and Strong (6), and Pennington, Snell, and Williams (7) respectively. Fruit juices and spent liquors were assayed directly, but yeast was first liquefied with one-fourth part of ethyl acetate, followed by at least 24 hours of autolysis at room temperature.

Nicotinic acid and biotin were determined by the *Lactobacillus arabinosus* method of Snell and Wright (8). *p*-Aminobenzoic acid was determined by use of the same microorganism (9). Fruit juices and spent liquors were assayed for nicotinic acid directly. The ethyl acetate treatment of the yeast sufficed for nicotinic acid and for *p*-aminobenzoic acid assays. Alkaline hydrolysis, which gives greatly enhanced *p*-aminobenzoic acid assays with many materials, generally gave small or no increases in assay values for torula yeast or spent liquor, but such hydrolysis was necessary to obtain maximum values on fruit juices. The ethyl acetate treatment of torula yeast was inadequate for liberation of biotin, although it increased assay values several times in comparison with those obtained with untreated fresh yeast. Further increases of about 5 times were obtained by autoclaving autolyzed yeast suspensions for 30 minutes in 3 N H_2SO_4. The acid autoclaving was also found to increase assay values of fruit juices and spent liquors. Because of relatively low biotin contents, removal of SO_4'' with $Ba(OH)_2 \cdot 8H_2O$ was necessary in the latter cases.

Pyridoxin was determined by the *Saccharomyces carlsbergensis* method of Atkin, Schultz, Williams, and Frey (10) on samples autoclaved in 0.05 N H_2SO_4 as recommended by these authors. Inositol was determined by a modification of the pyridoxin assay involving replacement of inositol in the basal medium by 0.10 p.p.m. of pyridoxin hydrochloride. Samples were prepared for assay by autoclaving for 30 minutes in 1 N H_2SO_4.

Choline determinations were made on powdered dried yeast by the reineckate precipitation method of Engel (11) and by the microbiological method of Horowitz and Beadle (12) using a mutant of *Neurospora crassa*.

Crystalline vitamins were used as standards in all cases. Thiamin values are expressed as thiamin chloride hydrochloride, pantothenic acid as calcium pantothenate, biotin as the free acid, and pyridoxin as the hydrochloride.

Some slight modifications of procedures were introduced in certain of the methods; these have been mentioned elsewhere (13). Numerous experiments on the applicability of microbiological assays to *Torulopsis utilis* have been made in this Laboratory, but need not be described here. It is believed that the results are reasonably reliable.

Results

Synthesis of Vitamins by Torula Yeast. Vitamin assays were made on inocula and fruit juices as well as on the crop yeast and spent liquors. These determinations permitted calculation of vitamin balances, includ-

TABLE I

Typical Vitamin Balance: Nicotinic Acid
(Run 12, Fermenter 3, peach juice)

Medium		
Total volume	ml.	1470
Vitamin concentration	γ/ml.	7.3
Total vitamin in medium	γ	10700
Seed yeast		
Total solids (dry weight basis)	g.	4.0
Vitamin concentration	γ/g.	570
Total vitamin in seed yeast	γ	2280
Yeast crop		
Total solids (dry weight basis)	g.	62.5
Increase over seed yeast	g.	58.5
Vitamin concentration	γ/g.	540
Total vitamin in yeast crop	γ	33800
Spent liquor		
Total volume	ml.	4100
Vitamin concentration	γ/ml.	0.82
Total vitamin in spent liquor	γ	3360
Vitamin balance		
Total vitamin in yeast crop and spent liquor	γ	37160
Total vitamin in medium and seed yeast	γ	12980
Total vitamin synthesized	γ	24180
Vitamin synthesized	γ/g. of yeast solids produced	415
Vitamin absorbed from medium	γ/g. of yeast solids produced	125
Vitamin absorbed from medium	% of total vitamin content in yeast	23

ing the extent of vitamin synthesis by the yeast, and of the extent of excretion of synthesized vitamins into the culture liquor or of the extent of absorption of vitamins from the medium. A typical vitamin balance is given in Table I. In general, the cake yeast on which vitamin assays

TABLE II

Summary of Vitamin Synthesis by Torulopsis utilis

Description	Range of Data	Dry Yeast Produced g. per 100 g. of sugar	Vitamin Content of Crop Yeast γ per g. of dry yeast	Net Vitamin Synthesis γ per g. of dry yeast produced	Net Vitamin Absorbed (+) from Medium or Excreted (−) into Medium γ per g. of dry yeast produced	Net Vitamin Absorbed (+) from Medium or Excreted (−) into Medium per cent of "total active vitamin"[1]
Thiamin						
Prune juice medium	4 standard runs	55 (51 to 62)	25 (11 to 42)	25 (10 to 47)	−1 (−3 to +2)	+2 (−10 to +7)
Prune juice medium	all runs (12)	53 (48 to 62)	25 (7 to 42)	27 (7 to 47)	−2 (−7 to +2)	+4 (−23 to +12)
Molasses medium	1 standard run	56	18			
5 other fruit juice media	7 standard runs	52 (45 to 59)	15 (9 to 27)	13 (8 to 23)	+2 (0 to +5)	+11 (0 to +23)
Riboflavin						
Prune juice medium	5 standard runs	55 (51 to 62)	47 (35 to 60)	78 (60 to 87)	−31 (−35 to −25)	−40 (−50 to −30)
Prune juice medium	all runs (13)	53 (48 to 62)	50 (35 to 61)	81 (60 to 103)	−34 (−48 to −25)	−42 (−54 to −30)
5 other fruit juice media	7 standard runs	52 (45 to 59)	38 (26 to 47)	54 (28 to 70)	−18 (−25 to −4)	−32 (−38 to −15)
Molasses medium	1 standard run	56	36	52	−35	−37
Nicotinic Acid						
Prune juice medium	6 standard runs	54 (51 to 62)	530 (455 to 630)	530 (430 to 625)	+4 (−130 to +94)	−2 (−21 to +16)
Prune juice medium	all runs (14)	53 (48 to 62)	555 (450 to 690)	570 (430 to 735)	−17 (−130 to +94)	−2 (−21 to +16)
5 other fruit juice media	7 standard runs	52 (45 to 59)	440 (375 to 540)	440 (410 to 570)	−39 (−154 to +125)	−8 (−33 to +23)
Molasses medium	1 standard run	56	610	505	+101	+17
Pantothenic acid						
Prune juice medium	5 standard runs	55 (51 to 62)	138 (106 to 175)	231 (124 to 344)	−94 (−214 to −26)	−37 (−62 to −21)
Prune juice medium	all runs (13)	53 (48 to 62)	143 (106 to 180)	238 (124 to 344)	−101 (−214 to −26)	−40 (−62 to −21)
5 other fruit juice media	7 standard runs	52 (45 to 59)	134 (109 to 159)	249 (198 to 353)	−113 (−224 to −47)	−43 (−66 to −29)
Molasses medium	1 standard run	56	141	222	−79	−36
Biotin						
3 fruit juice media	3 standard runs	53 (45 to 62)	1.33 (1.08 to 1.50)	1.31 (1.04 to 1.48)	+0.01 (0 to +0.02)	+0.6 (−0.1 to +1.2)
Molasses medium	1 standard run	56	1.06	0.99	+0.05	+5
Pyridoxin						
3 fruit juice media	3 standard runs	53 (45 to 62)	36 (29 to 47)	37 (27 to 56)	−1 (−8 to +5)	0 (−15 to +15)
Molasses medium	1 standard run	56	36	29	+7	+20
p-Aminobenzoic Acid						
3 fruit juice media	4 standard runs	53 (45 to 62)	23 (16 to 40)	68 (42 to 94)	−45 (−64 to −26)	−66 (−79 to −56)
Molasses medium	1 standard run	56	16	20	−4	−22

AVERAGES

Thiamin	19 runs	53	22	22	−0.5	+2
Riboflavin	21 runs	53	44	71	−29	−38
Nicotinic Acid	22 runs	53	535	530	−18	−3
Pantothenic Acid	21 runs	53	139	241	−104	−41
Biotin	4 runs	54	1.26	1.23	+0.02	+1
Pyridoxin	4 runs	54	36	35	+1.0	+4
p-Aminobenzoic Acid	5 runs	54	21	56	−37	−55

[1] By "total active vitamin" is meant the net fraction of the total vitamin content of the culture system that has been synthesized or that has been absorbed from the medium by the growing yeast. It excludes the vitamin content of the inoculum and the net non-absorbed vitamin originally present in the medium. The total active vitamin is equivalent to the total vitamin content of the yeast produced or to the total vitamin synthesis in accordance with the following relations:

(a) Vitamin synthesis + net vitamin absorption by yeast = vitamin content of yeast produced.

(b) Vitamin content of yeast produced + net vitamin excretion from yeast = vitamin synthesis.

were made was not washed. The errors arising from the vitamin content of occluded spent liquor were small, however, and have been neglected.

The summarized results of the vitamin balance experiments are given in Table II. In addition to vitamin assays of yeast crops and determinations of vitamin synthesis, the shifts of vitamins in the culture system by absorption of vitamins from the medium by the yeast or by excretion of vitamins from the yeast into the medium are given in the table. These shifts of vitamins in the system were calculated to a common base so that absorption and excretion could be considered as inverses and averaged. This was desirable since in the cases of some vitamins a net absorption might take place in one run and a net excretion in a later similar run. Of necessity the vitamin balances neglect vitamin destroyed in the culture medium or metabolized within the yeast cells.

Almost 100 individual vitamin balances were calculated. Some 20 individual fermentations were involved in the cases of certain vitamins. These individual fermentations were conducted in groups of at most 6 at a time. The various groups were, in some instances, run at widely separated time intervals. It was noted that in most cases the variations in vitamin content and synthesis between groups of fermentations were greater than between individual fermentations in a given run. In most cases this makes the difference in results obtained with prune juice, with press juices of five other fruits (apples, pears, peaches, raisins, and figs), and with molasses of doubtful significance.

The errors of the vitamin assays used are generally considered to vary up to 5 to 15 per cent, depending on the method used. It is clear that such relatively small errors fail to explain the wide ranges of vitamin content and synthesis found for certain vitamins in Table II. It is becoming increasingly evident that numerous factors, both internal and external, affect the extent of vitamin synthesis by microorganisms. It may be noted that the greatest range in our data is found in the case of thiamin. Some factors affecting thiamin content of yeast are discussed later.

Most of the fermenter runs were conducted under the conditions outlined above and also in greater detail in Table I of the article by Stubbs, Noble, and Lewis (2). These runs are referred to in Table II as "standard runs." Various modifications of the standard run were made, such as use of smaller culture volumes, addition of pyruvic acid, variations in inocula, and subjection of the crop yeast to a supplemental anaerobic fermentation in fresh medium. The average results of the modified and standard runs given for 4 vitamins in Table II on the lines marked "all

runs" closely resembled those of the standard runs, except that supplemental anaerobic fermentation increased thiamin synthesis and thiamin retention in the spent liquor, as will be discussed later.

Choline assays on two samples of torula yeast grown on prune sirup and molasses media in pilot-plant scale equipment showed 3.5 and 2.9 γ, respectively, of choline per gram of dry yeast by the reineckate method and 3.4 and 2.9 γ per gram of dry yeast by the Neurospora method (see Acknowledgment).

Two inositol assays carried out on torula yeast grown on fruit substrates gave contents of 2850 and 2500 γ per gram of dry yeast. A number of inositol assays on torula yeast grown on sucrose indicated inositol synthesis ranging from 300 to 3400 γ per gram of dry yeast (13).[1]

Significance of Naturally Occurring Vitamins in Fruits. It is of interest to evaluate the significance of naturally occurring vitamins of fruits with respect to the production of yeast and fermented feeds. While such vitamins might assist in supplying the needs of vitamin-requiring bakers' and brewers' yeasts, torula yeast does not require an external supply of vitamins for the production of high yeast crops. Nevertheless, the presence of these vitamins makes possible absorption of vitamins by the yeast, on the one hand, and recovery of vitamins from the medium by other means, on the other.

Some comparisons of the relative amounts of the vitamins available in fruits and the amounts obtained through synthesis by torula yeast are given in Table III. The estimations were made by relating the vitamin contents of fruits to their sugar contents and also to the amounts of vitamins that could be produced by the growth of torula yeast on similar amounts of sugar. The calculations indicate, for example, that the amounts of *p*-aminobenzoic acid occurring naturally in fruits are insig-

[1] Dr. Beadle of Stanford University obtained inositol assays, using an inositol-requiring mutant of *Neurospora*, on the two torula yeast samples submitted to Dr. Horowitz for choline determinations (correspondence with Dr. Horowitz). These were 3600 and 3400 γ, respectively, of inositol per gram of dry yeast for the samples grown on prune sirup and on molasses media, using yeast samples which had been hydrolyzed for 2 hours in 3 per cent H_2SO_4 in the autoclave. Dr. Beadle found this hydrolysis method to be inadequate for brewers' yeast, which gave a value of 2700 γ of inositol per gram as compared with 4800 γ of inositol per gram for the same yeast hydrolyzed by refluxing for 6 hours in 18 per cent HCl. There is, accordingly, reason to believe that our inositol assays are low, and that the wide variations in inositol synthesis by torula yeast reported by Lewis (13) may instead represent variation in the ease of hydrolysis of bound inositol of the yeast.

TABLE III

Comparisons of Vitamins Available in Fruit Juices and Amounts Synthesized by Torulopsis utilis Expressed in Terms of Fermentable Sugar

Vitamin Content of Fruits[1]	Apples	Pears	Peaches	Plums	Prunes	Oranges	Grapes	Raisins	Figs	Approximate Range for All Fruits (%)
Thiamin										
γ per gram of sugar	5[2]	6	3	9	6	12	3	3	1.5	
% of average synthesis	50	60	30	90	60	120	30	30	15	20 to 100
Riboflavin										
γ per gram of sugar	2	6	3	5	10	5	3	1.5	1.5	
% of average synthesis	5	15	8	12	25	12	8	4	4	4 to 25
Nicotinic Acid										
γ per gram of sugar	15	20	50	30	60	20	50	9	(12)[3]	
% of average synthesis	6	8	20	12	25	8	20	4	(5)	4 to 25
Pantothenic Acid										
γ per gram of sugar	6	6	12	—	2	(50)	—	2	—	
% of average synthesis	5	5	10	—	2	(40)	—	2	—	2 to 10
Biotin										
γ per gram of sugar	0.08	0.02	0.08	—	(0.01)	0.15	—	0.08	—	
% of average synthesis	8	2	8	—	(1)	15	—	8	—	2 to 15
Pyridoxin										
γ per gram of sugar	(3)	(4)	1.5	—	(4)	8	—	(1.5)	—	
% of average synthesis	(15)	(25)	10	—	(25)	50	—	(10)	—	10 to 50
Inositol										
γ per gram of sugar	(2500)	(2500)	5000	—	(6000)	(25000)	—	(2500)	—	
% of average synthesis	(250)	(250)	500	—	(600)	(2500)	—	(250)	—	250 to 2500
p-Aminobenzoic Acid										
γ per gram of sugar	0.2	0.8	1.5	—	(0.4)	—	—	(0.15)	—	
% of average synthesis	1	3	6	—	(1.5)	—	—	(0.5)	—	0.5 to 5

[1] Calculated from values reported in the literature or determined in this laboratory.

[2] Sample calculation: From values in the literature it appears that apples contain about 0.5 p.p.m. of thiamin and 10 per cent of sugar, or 5γ of thiamin per gram of sugar. One gram of sugar is sufficient to produce about 0.5 gram of dry torula yeast, during the propagation of which about 10γ of thiamin would be produced (22γ per gram of yeast on the average, from Table II). The amount originally present in the fruit then represents approximately 50 per cent of that which would be expected from the growth of torula yeast on apple sugar. The rough nature of the calculations is obvious, but they indicate marked differences among the various B vitamins in the relations between their distribution in fruits and their synthesis by torula yeast.

[3] The values in parentheses are derived from unreplicated or otherwise less reliable data.

nificant in relation to the amounts synthesized by yeast, while on the other hand inositol synthesis by yeast is small when compared with the amounts already present in fruits.

TABLE IV

Effect of Delayed Separation on Vitamin Content of Torulopsis utilis

Vitamin Distribution	Riboflavin	Pantothenic Acid	Nicotinic Acid	p-Aminobenzoic Acid
	γ/l. of culture	*γ/l. of culture*	*γ/l. of culture*	*γ/l. of culture*
In yeast, immediate separation	216	640	3650	110
In yeast, delayed separation	220	605	3770	72
In culture liquor, immediate separation	225	190	420	322
In culture liquor, delayed separation	223	160	440	367
In culture liquor, from medium	50	60	420	7
Total, immediate separation	441	830	4070	432
Total, delayed separation	443	765	4210	439
Fraction of total vitamin synthesized retained by yeast (immediate separation)	% 55	% 83	% 100	% 26
Fraction of total vitamin synthesized retained by yeast (delayed separation)	56	86	100	17
Change in vitamin content of yeast on standing	+2	+4	0	−35

Yeast crop was 6.0 g. of dry yeast per liter of prune juice medium after 7 hours of aeration. The culture was divided into two lots, from one of which the yeast was immediately separated and from the other of which the yeast was separated after standing 18 hours at 2°C.

By reference to Table II it may be seen that in some cases appreciable absorption of vitamins originally present in the medium was obtained. While the effects were not large in the present experiments, it is possible that in certain cases vitamins originally present may be able to contribute materially to the vitamin contents of fermented feeds prepared from fruit wastes. It must be emphasized, however, that the sugar content

would be the principal reason for using fruit wastes for torula yeast production; the vitamins, like the inorganic constituents and the non-carbohydrate fermentable material, would be of secondary importance.

Retention of Synthesized Vitamins by Torula Yeast. Table II shows that relatively large fractions of the riboflavin, pantothenic acid, and especially the *p*-aminobenzoic acid were excreted into the medium during the 6 to 8 hours of fermentation or during the period following fermentation in which the yeast remained in contact with the culture liquor. This period customarily consisted of overnight storage at 2° C. An experiment concerned with this point is summarized in Table IV. It may be seen that of the four vitamins studied only *p*-aminobenzoic acid decreased significantly in quantity on standing on contact with the fermentation liquor. One must then conclude that the excretion of riboflavin and pantothenic acid, as well as of *p*-aminobenzoic acid, occurred mainly during the propagation period.

Discussion

The quantities of certain B vitamins contained in torula yeast have previously been reported. Fink and Just (14, 15), in an extensive series of investigations, found that the thiamin content of yeast depends largely on the thiamin contents of the substrate and of the inoculum, on the extent of propagation relative to the available thiamin, and on the type of metabolism during propagation. Propagation under aeration, as is usual for torula and bakers' yeasts, results in marked losses of thiamin from the medium and low contents in the yeast. A fermentative type of metabolism, on the other hand, as is usual for brewers' yeast, favors the uptake of thiamin from the medium without loss, and also the synthesis of thiamin by torula yeast. Torula, bakers', and brewers' yeasts when propagated under similar conditions contain similar amounts of thiamin, indicating that the species of yeast is not a primary factor in determining the thiamin content of yeast.

Fink and Just report an average thiamin content of torula yeast grown under aeration of 20 γ per gram of dry yeast. This is very close to our average value of 22 γ per gram (Table II). They state that the thiamin content of torula yeast may be increased if it is allowed to undergo a fermentation without aeration or if the substrate is supplemented with thiamin. We have verified the beneficial effect of supplemental non-aerated fermentation on thiamin synthesis, in that supplemental overnight fermentation without aeration increased the thiamin synthesis of

torula yeast cultured in the usual fashion on prune juice medium from 15 γ per gram up to 30 γ per gram of yeast. It is clear that the conditions of culture used in this work to obtain high yeast yields on fruit juice media are not conducive to high thiamin content or synthesis. Torula yeast grown under such conditions, like bakers' yeast, is markedly poorer in thiamin than brewers' yeast.

It is well-known (16, 17, 18) that bakers' and brewers' yeasts are capable of converting the pyrimidine and thiazole moieties of thiamin into thiamin with a high degree of efficiency. Van Lanen, Broquist, Johnson, Baldwin, and Peterson (17) found that torula yeast posesses the ability to convert an equimolar mixture of thiazole and pyrimidine into thiamin, although with a lower efficiency than did bakers' yeast under their conditions.

Scheunert and Wagner (19) found the vitamin B_2-complex potency by rat assays of torula yeast to be similar to that of bakers' yeast. Our torula yeast averaged 44 γ of riboflavin per gram of dry yeast, which may be somewhat lower than the usual values for bakers' and brewers' yeasts.

Fink and Just (4) determined the nicotinic acid content of torula yeast grown on a wide variety of substrates. The values varied from 343 to 453 γ per gram of dry yeast with an average of about 375 γ per gram. Brewers' yeast contained about 510 γ per gram, while bakers' yeast contained about 300 γ per gram. Our assays of torula yeast have run definitely higher, with an average of about 535 γ per gram of yeast.

Data from other sources as to the content of other B vitamins in torula yeast are lacking. The pantothenic acid, biotin, and pyridoxin contents of torula yeast grown on fruit juice media appear to be similar to the relatively few values in the literature for commercial yeasts.

It is also interesting to compare the vitamin content of torula yeast with that of various bacteria. Lee and Burris (20) gave vitamin assays for *Azotobacter vinelandii* grown in pilot-plant-scale equipment. This bacterium is characterized by exceptionally high contents of riboflavin and biotin (325 and 3.5 γ per gram respectively). The thiamin content was higher than for torula yeast, while equivalent amounts of nicotinic and pantothenic acids were found. Woods, *et al.* (21) gave assays for several bacterial species, including *Aerobacter aerogenes*, *Serratia marcescens*, *Pseudomonas fluorescens*, and *Clostridium butylicum*. When calculated to the dry basis it is found that the contents of most of the B vitamins are similar to those for torula yeast, the most marked difference being a higher biotin content in the bacteria.

It is evident that while certain microorganisms may contain more of individual vitamins than does *Torulopsis utilis*, when the high efficiency of conversion of fermentable substrates into yeast substance is considered, *Torulopsis utilis* is an outstanding producer of the vitamin B complex.

Summary

Torulopsis utilis grown in high yields on fruit juice substrates was assayed for members of the B group of vitamins. The vitamin content was found to be roughly comparable to that of bakers' and brewers' yeasts, and certain other microorganisms. When the high yields of *Torulopsis utilis* obtainable from cheap substrates are considered, it appears that this yeast is an outstanding vitamin producer. Significant amounts of a number of the vitamins are also found in the culture liquors.

Acknowledgment

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Effect of Heat on Protyrosinase

Heat Activation, Inhibition, and Injury of Protyrosinase and Tyrosinase*

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Heat activation of protyrosinase from the diapause egg of the grasshopper, *Melanoplus differentialis*, has been previously described (1). Further studies along these lines have yielded additional data concerning certain physicochemical changes during such activating phenomena. The present paper deals with certain of these changes in some detail.

Material and Method

Protyrosinase was obtained from the diapause egg of the grasshopper, *Melanoplus differentialis*, as already reported (2). Samples of the protyrosinase were standardized by activation with aerosol OT so as to catalyze the uptake of 100 cu. mm. of O_2 by 0.3 ml. of 0.4 per cent tyramine-HCl at 25°C. in 10 minutes. For convenience, this will be referred to as 100% activation of the protyrosinase. Warburg manometers were used in a manner as previously noted (3).

In addition to the use of phosphate buffers, imidazole[2] was also employed. The pH values were recorded with a glass electrode (Leeds and Northrup, No. 7661 Universal pH potentiometer).

Exposures to temperatures were obtained by immersion of test tubes containing the experimental mixtures in large volumes of H_2O manually maintained at the desired temperatures (±1.0°C.). Variations in the normal procedures are fully indicated in the description of individual experiments.

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[1] Now in active service with the A.U.S.

[2] The authors are indebted to Dr. E. D. Warner of the Department of Pathology for generous samples of imidazole. All other samples of imidazole were obtained from Eastman Kodak Company. For methods of preparation and use, see E. T. Mertz, and Owen, C. A., *Proc. Soc. Exptl. Biol. Med.* **43**, 204 (1940).

Heat Activation of Protyrosinase

The marked resistance of protyrosinase to temperatures usually lethal to most enzymes has previously been noted (1). Between 55° and 60°C. practically all respiratory enzymes in the intact grasshopper egg are destroyed, and curiously it is at this point that heat activation of protyrosinase in the intact egg begins (4). Similar data have recently been obtained for the activation by heat of protyrosinase extracts. Results of typical experiments are graphically shown in Fig. 1 where effects of

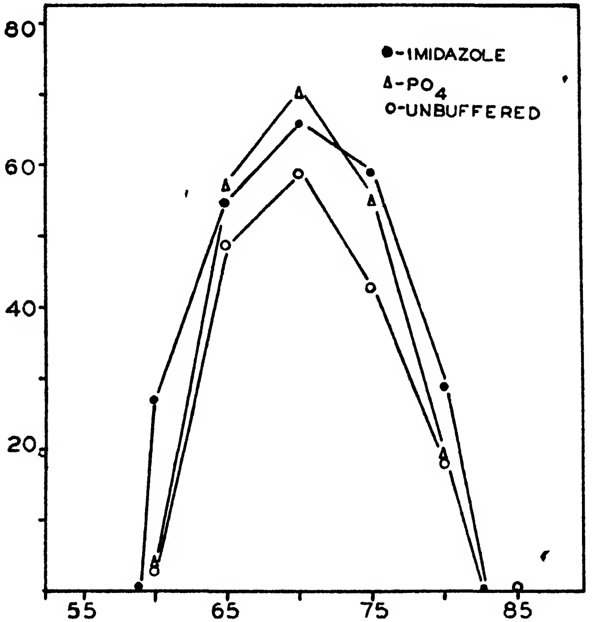

FIG. 1

Shows the Activating Effect of 10 Minute Exposures of Protyrosinase Extracts to Different Temperatures

Extracts, when subjected to temperatures, were buffered with imidazole (pH 6.6), phosphates (pH 6.6), and unbuffered (pH 6.0). All determinations of activity were carried out in buffered solutions (pH 6.6) at 25°C. Abscissa, temperature in degrees centigrade. Ordinate, percentage activity of buffered extracts when compared with aerosol OT activated as 100 per cent.

exposures of 10 minutes on the activation of protyrosinase at temperatures ranging from 55° to 85°C. are presented. As the temperature is increased a maximum activation of approximately 70% is reached at

70°C. Above this temperature marked destruction of the tyrosinase thus produced occurs until at approximately 83° C. it is completely destroyed.

Results of experiments carried out at a constant pH value of 6.6 with phosphate or imidazole buffers show no marked qualitative differences in heat activation from the unbuffered extracts (Fig. 1). Quantitative differences, however, are apparent. Buffered protyrosinase extracts show a slightly greater activation by heat than unbuffered ones. The extent to which these differences in response are due to pH and to buffer effects as such has also been investigated. Results of a series of experiments on the effects of exposures to 70°C. for 10 minutes with phosphate and imidazole buffers at different pH values are graphically summarized in Fig. 2. Generally, phosphate buffers at pH values ranging from 5.5 to 7.0 show no appreciable differences in the activation of protyrosinase when subjected to 70°C. for 10 minutes. The relative amounts of phosphates employed up to 2 times that normally used also seem of little importance for the reaction. Imidazole, when used as buffer on the other hand, always gives a slightly lower activation of the protyrosinase at corresponding temperatures. A tendency for higher rates of activation of the enzyme to occur with imidazole at higher pH values is indicated. Varying the amounts of imidazole employed, as for phosphates, does not in itself seem to alter the results. It seems, therefore, that in the action of heat on the protyrosinase the effects of pH and buffer on the activation of the enzyme are in themselves of minor significance and that the effects noted are largely those produced by the temperature alone.

The reaction of nonheat-treated protyrosinase to phosphate and imidazole buffers is also of interest. When protyrosinase is treated with phosphate or imidazole buffers at different pH values (6.2–7.4) and then activated with aerosol OT at 25°C. a slightly greater activation is produced at the higher pH values (Fig. 3). If the buffers are added to protyrosinase previously activated by aerosol OT, the subsequent action of the enzyme is similar to the protyrosinase treated one except that it is somewhat lower in value. This perhaps indicates differences in susceptibility of the inactive (protyrosinase) and active (tyrosinase) forms to the phosphate and imidazole buffers as such.

Activation, Injury, and Inhibition of Protyrosinase at Higher Temperatures

Inasmuch as activation of protyrosinase by heat at controlled pH values seems a rather constant phenomenon, it becomes of some interest to analyze the various changes produced in the enzyme extract during the course of such a reaction.

Maximum activation of standard samples of protyrosinase at 25°C.

are obtained at pH 6.6 upon the addition of adequate amounts of aerosol OT (5). This fact has been utilized in dealing with the effects of heat on protyrosinase since after heating and determining the amount of tyro-

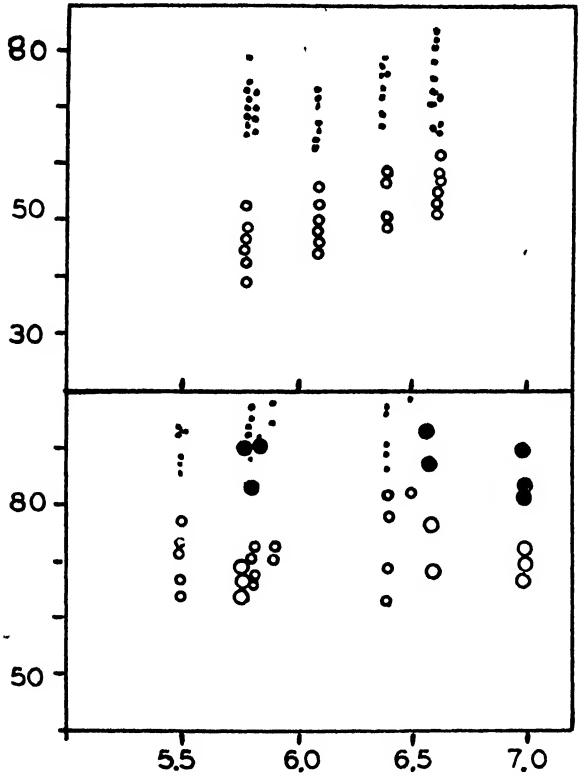

FIG. 2

Shows the Activating Effect of 70°C. for 10 Minutes on Protyrosinase Extracts when Buffered with Imidazole and Phosphates at Different pH Values

Upper part, imidazole; lower, phosphates. Abscissa, pH; ordinate, percentage activity of buffered extracts when compared with aerosol OT activated (pH 6.6) as 100%. Open circles, activity produced by heat exposure; dots, activity produced when aerosol OT is added to heat treated sample. Large circles and dots are for results obtained when twice the normal amount of buffer was employed. All determinations of activity were carried out in buffers (pH 6.6) at 25°C.

sinase thus produced, the addition of aerosol OT then activates the remaining nonheat-activated fraction. The relative effects of heat as an activator as well as an injurious agent are thus readily obtained. In Fig. 4

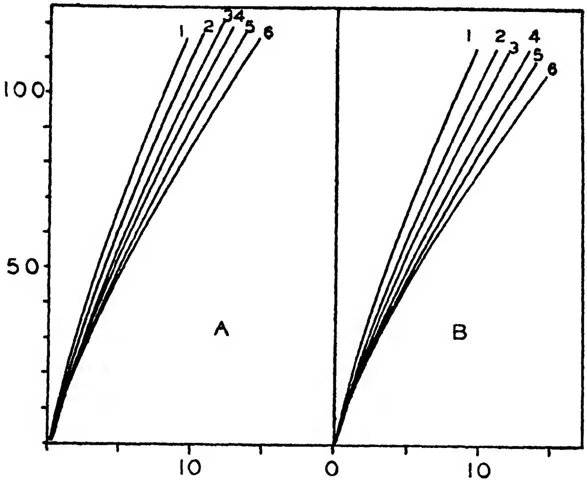

Fig. 3

Shows the Effect of pH, Produced by Phosphates and Imidazole, on the Activity of Protyrosinase and Tyrosinase

A, phosphates. B, Imidazole. Abscissa, time in minutes. Ordinate, oxygen uptake in cu. mm. Curve 1, protyrosinase at pH 7.4; Curve 2, tyrosinase at pH 7.4; Curve 3, protyrosinase at pH 6.8; Curve 4, tyrosinase at pH 6.8; Curve 5, protyrosinase at pH 6.2; Curve 6, tyrosinase at pH 6 2. For further description, see text.

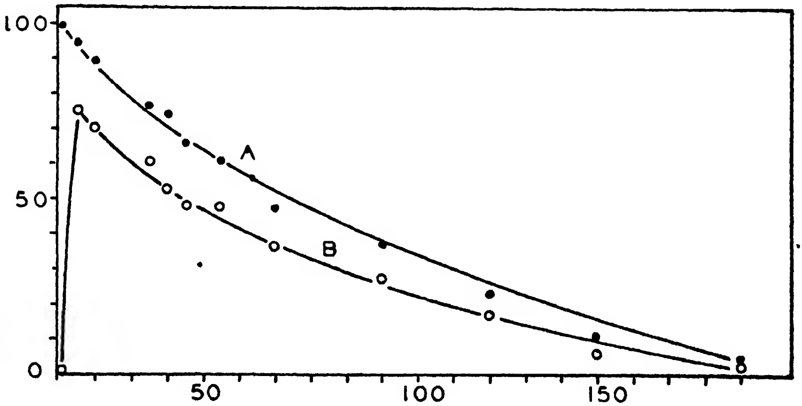

Fig. 4

Shows the Effect of Exposure to 70°C. on the Activation of Protyrosinase

A, activation due to heat plus aerosol OT; B, activation due to heat alone. Abscissa, time in minutes. Ordinate, per cent activity in terms of aerosol OT activation as 100%.

are graphically shown the effects of 70°C. for varying periods on the activation of protyrosinase at constant pH (6.6) with phosphate buffers. A maximum activation of approximately 75% is obtained after an exposure of 5 minutes. Longer exposures produce decreasing amounts of tyrosinase and increasing amounts of injury. With an exposure of approximately 190 minutes at 70°C. all the enzyme is destroyed. When aerosol OT is added to the heat-activated extract (at 25°C.) an increase in the activation over that produced by the heat alone occurs (Fig. 4).

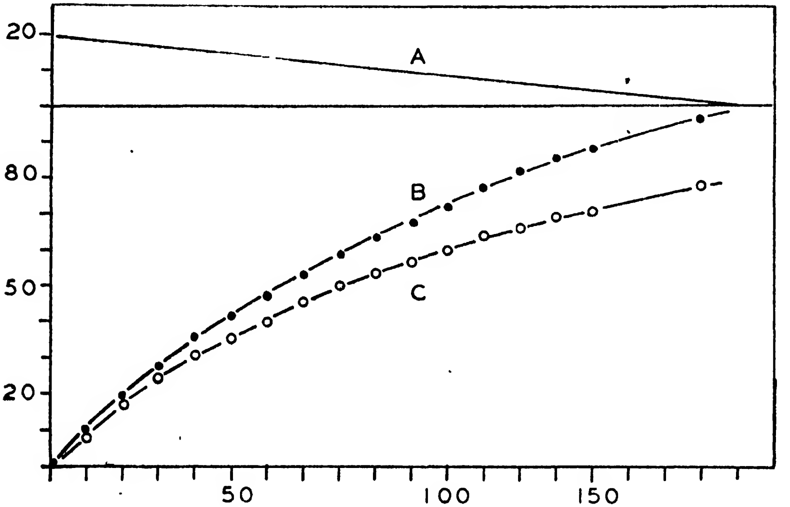

Fig. 5

Shows the Effect of Exposure to 70°C. on Inhibition (A), Total Reduction in Activity (B), and Injury to Enzyme (C)

Abscissa, time in minutes. Ordinates, at top, percentage inhibited protyrosinase; at bottom, percentage injury. For further description, see text.

This increase in activation is presumed to represent the protyrosinase inhibited (?) but not destroyed by the heat exposure in question. As the heat exposure is increased this amount of inhibited protyrosinase decreases and eventually disappears due to its complete destruction. It is thus possible to construct from these values curves indicating first, the relative amounts of inhibited protyrosinase; second, the total injury plus inhibition; and third, the tyrosinase injury due to the varying lengths of exposure to 70°C. (Fig. 5). From an inspection of these, it seems evident that heat-activated tyrosinase as such is probably more

susceptible to exposure to 70°C. than is protyrosinase over a considerable range of exposures. The nature of the heat-inhibited protyrosinase fraction is at present not clear but that it does not occur in tyrosinase produced by other activators will be shown below. It is of some interest to recall in this connection that in the shaking of protyrosinase extract, tyrosinase is produced and at the same time inhibited or inactive products are formed (6).

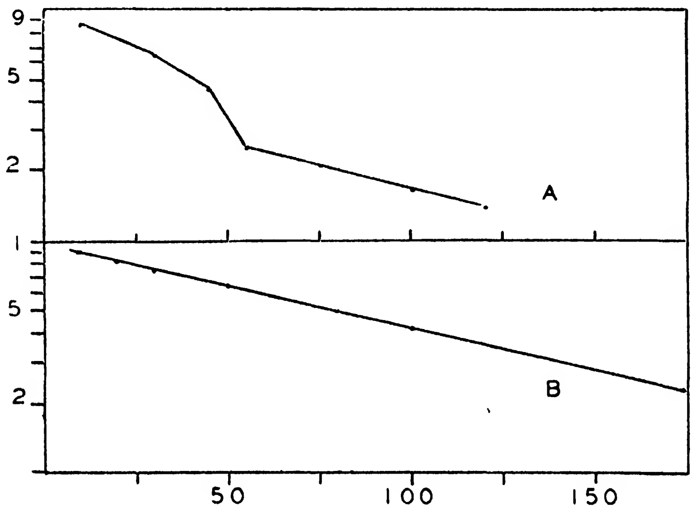

FIG. 6

Shows Percentage Injury Due to Exposure of Sodium Dodecylsulfate Activated (A) as well as Heat Activated (B) Protyrosinase to 70°C. when Plotted on Semilogarithmic Paper

Abscissa, time in minutes. Ordinate, per cent injury.

Heat injury or inactivation of the tyrosinase produced from the heat activated protyrosinase seems to follow the course of a unimolecular reaction as indicated in Fig. 6.

Effect of Temperature on Tyrosinase Produced by Different Activators

The relatively high resistance to temperature of tyrosinase produced from heat-activated protyrosinase led to a comparison of this property with tyrosinase produced by other activators such as aerosol OT, sodium dodecylsulfate, sodium oleate, and urea.

With uniform samples of protyrosinase, comparisons of the relative

activating powers of different activators are possible. Aerosol OT, sodium dodecylsulfate, and sodium oleate produce, within limits, approximately 100% activation with no significant injury while urea produces but 80% activation and some 20% injury (1). This difference in activation is probably associated with a toxic or some secondary reaction of the activator with the enzyme. The catalytic properties of the tyrosinase, however produced, are similar. Its resistance to injury by heat, varies considerably.

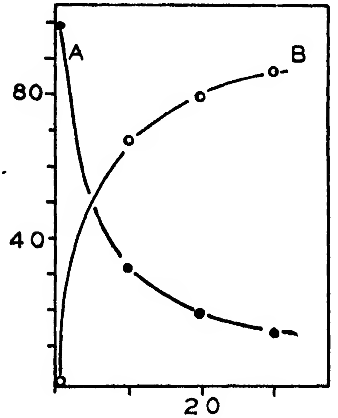

FIG. 7

Shows Effect of 65°C. on Activity (A) and Destruction (B) of Aerosol OT Activated Protyrosinase

Abscissa, time in minutes. Ordinate, per cent activity and destruction.

Samples of protyrosinase, treated with different activators to produce complete or maximum activation at 25°C., were exposed to 70°C. for varying periods of time and injury to the tyrosinase then determined. Aerosol OT activated tyrosinase was quickly destroyed at 70°C. and at 65°C. showed marked injury (Figs. 7 and 8). Exposure of 10 minutes at 65°C. produced more than 60% injury. Urea activated tyrosinase, on the other hand, is more resistant and at 70°C. for 15 minutes suffers approximately 60% injury (Fig. 8). Sodium dodecylsulfate activated tyrosinase is next in the series of resistance, then heat-activated tyrosinase and finally sodium oleate activated enzyme. As indicated in Fig. 8, a marked and graded difference in susceptibility to temperature occurs in the tyrosinase produced by these activators. The series arranged in order of susceptibility is as follows:

Aerosol OT > urea > sodium dodecylsulfate > heat > sodium oleate.

A curious and somewhat anomalous change in the heat resistance of sodium oleate activated tyrosinase has been noted. Freshly prepared sodium oleate produces tyrosinase which is quite similar to sodium dodecylsulfate or heat-activated tyrosinase. When the same sodium oleate solution is allowed to stand at 25°C. for a period and then used as activating agent a marked resistance to heat is found in the tyrosinase thus produced. Similar results are obtained when either phosphate or imidazole buffers are employed. The marked instability of sodium oleate

solutions may well account for its production of tyrosinase of such varying resistance to temperature.

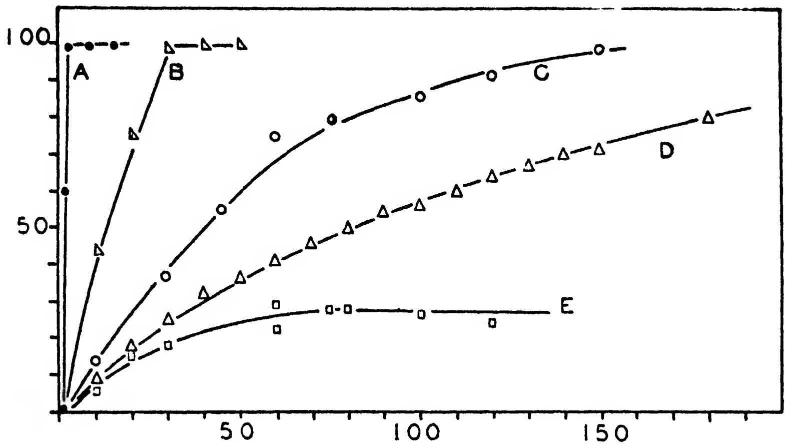

Fig. 8

Shows Effect of 70°C. on Injury to Protyrosinase Activated by Different Reagents

Abscissa, time in minutes. Ordinate, per cent injury. A—aerosol OT activated; B—urea; C—sodium dodecylsulfate; D—70°C.; E—sodium oleate.

Discussion

The above results suggest that the conversion or activation of protyrosinase to tyrosinase is associated with molecular rearrangements or conversions and that these modified protein molecules possess physical properties related to or characteristic of the type of change thus produced. Recent information for other proteins (sera, egg albumin, viruses, etc.) seems to suggest such possibilities (7, 8, 9). For tyrosinase produced by different activators the catalytic parts of the molecule must in all cases be similar but side chains or other parts of the molecule influenced specifically by the activator in question may well be of quite different natures. These differently arranged side chains confer upon the enzyme molecules (proteins) different physico-chemical properties among which is a marked variation in susceptibility to temperature. It is also reasonable to assume that compounds are likewise formed by the enzyme-activator complex as indicated for egg albumin by the recent work of Lundgren, Elam, and O'Connell (8). These complexes in turn may also contribute to these marked differences in the physico-chemical properties.

The action of heat on the different types of complexes is quite irregular as shown by the fact that heat inactivation of tyrosinase, produced by exposure of protyrosinase to 70°C., closely follows the course of a unimolecular reaction (Fig. 6) while for tyrosinase produced by other activators the resulting log time curves deviate from a straight line. Results obtained for heat inactivation of viruses and other enzymes also seem to vary to a great degree (9).

Summary

1. Protyrosinase extracts, obtained from the egg of the grasshopper, *Melanoplus differentialis*, have been subjected to a series of temperatures ranging from 55° to 85°C and their activation followed.

2. Temperatures from 55° to 70°C. produce maximum activation while exposures to higher temperatures result in injury to the tyrosinase thus produced. At 83°C. the enzyme is destroyed.

3. Tyrosinase, produced by different activators, although showing similar catalytic properties, varies to a great degree in its resistance to temperature. The order of susceptibility to temperature for the activators tested is:

Aerosol OT > urea > sodium dodecylsulfate > heat > sodium oleate

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Availability of Vitamins in Foods and Food Products

I. Utilization of Thiamin in Brewers' Yeasts*

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With the Technical Assistance of Leslie Easterling

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Received April 24, 1944

Introduction

In protein and mineral metabolism the importance of absorption and retention has been recognized for many years. As a matter of fact, fecal and urinary excretions of nitrogen provide the necessary information for biological value of proteins (1). Similar technique discloses what proportions of the total iron (2) or calcium (3) intake are available. Information is, however, lacking on the availability of vitamins in foods; also nothing is known as to whether different methods of dehydration or processing of foods influence the absorption or retention of vitamins. For this reason, a series of studies were begun in 1943 in this department along this line.

In 1933, Walker and Nelson (4) found that the thiamin content of fresh yeast doubled on drying. These observations were confirmed by Parsons and Collord in 1942 (5) who demonstrated by balance studies that there was a much greater elimination of thiamin in feces of human subjects fed fresh yeast than when they were fed an equal amount of fresh yeast previously subjected to boiling temperature. That thiamin in dehydrated brewers' yeasts is well utilized has become evident from our investigation, the results of which are submitted in summarized form in this communication.

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Experimental

(a) Methods

Thiamin utilization in three brands of brewers' yeasts was studied in 72 albino rats. Balance experiments were conducted in three sets of animals, 24 of each set for each brand of yeast, according to methods previously described (6). For thiamin determinations we used a modification of the method of Hennessy and Cerecedo (7) introduced by the research laboratories of Merck and Co. (8). For the riboflavin content of feces, tissues, and endocrine glands, we followed the procedures of Conner and Straub (9), and for the riboflavin content of urine we used our recent modification (10) of the method of Hodson and Norris for determining the riboflavin content of foodstuffs (11).

The thiamin content of the three brands of brewers' yeast we used were 440 γ/g., 670 γ/g., and 160 γ/g. and were designated as yeasts A, B, and C respectively. The animals were fed a purified synthetic diet of the following composition: Casein (vitamin-free, Smaco), 18; cellu-flour, 2; salts No. 1 (12), 4; butter fat, 10; and cerelose, 66. The vitamin supplements were given daily separately from the ration, as follows: 20 γ riboflavin, 20 γ pyridoxin, 6 mg. choline chloride, and 200 γ calcium pantothenate. The brewers' yeasts were administered daily in petri dishes sprinkled with cerelose which insured complete consumption. As a source of vitamins A and D, three drops of halibut liver oil were given once weekly to each animal.

(b) Results

Yeast A

In this series there were 12 male and 12 female rats. They were started on experiments when 46 days old, weighing 100 to 125 g. each. The metabolism study was conducted for 28 days, the first 7 days of which constituted the thiamin depletion period. The data secured during the thiamin depletion period were subtracted from the total urinary and fecal thiamin excretions secured during the subsequent metabolism periods, for the calculations of the absorption and retention of this vitamin furnished by the brewers' yeasts. Two doses of yeast A were administered daily—50 mg. and 150 mg. to the males, and 100 and 200 mg. to the females, in two metabolism periods of 14 and 7 days respectively, furnishing 22 to 88 γ thiamin daily to each animal. It will be noted from Table I that the thiamin in this yeast which contained 440γ/g. was well absorbed and utilized by the males in daily amounts of 50 and 150 mg. yeast supplying 22 to 66 γ thiamin. The females showed equal efficiency in both absorption and utilization of 100 mg. yeast providing 44 γ thiamin. However, on 200 mg. yeast containing 88γ thiamin there was a decline of 7% in absorption and utilization but still allowed 93% efficiency.

Yeast B

In this series there were 24 male rats which were started on experiments when 65 days old, weighing 110 to 135 g. each. Balance experiments on yeast B, containing 670 γ/g. were conducted for 49 days in three metabolism periods: (1) 7 days of thiamin depletion; (2) 14 days maintenance which was secured by controlling feed intake; (3) 28 days of growth by allowing feed *ad libitum.* This set of animals was divided into 4 groups: (A) 6 rats were given 50 mg. yeast daily furnishing 33.5 γ thiamin; (B) an equal number of animals were allowed 33.5 γ pure crystalline synthetic

TABLE I

Availability of Thiamin in Brewers' Yeasts[1]

Yeast	Number of animals and sex	Metabolism period	Daily yeast intake	Daily thiamin intake	Total thiamin intake	Change in body weight	Thiamin excreted in feces[2]	Thiamin absorbed		Thiamin excreted in urine[2]	Thiamin retained	Thiamin utilized
		days	*mg*	γ	γ	*g.*	γ	γ	%	γ	γ	%
A	12 ♂'s	14	50	22	308	+30.0	29.3	278.7	90.5	0.77	277.9	99.7
	12 ♂'s	7	150	66	462	+19 3	40.6	421.4	91.2	5.91	415.5	98.6
"	12 ♀'s	14	100	44	616	+25 0	66.2	549.8	89.2	37.5	546.0	99.3
	12 ♀'s	7	200	88	616	+7.0	104.8	511.2	82.9	37.5	473.5	92.6
B	6 ♂'s	14	50	33.5	469	±0.0	34.7	434.3	94.7	0.0	434.3	100 0
	6 ♂'s	14	—	33.5	469	±0 0	42.6	426.4	92.9	4.3	422.1	99.0
	6 ♂'s	14	100	67.0	469	+1.5	82.0	387.0	82.5	9.3	377.7	97.2
	6 ♂'s	14	—	67.0	469	+0.7	35.4	433.6	94.7	25.7	428.1	98.8
"	6 ♂'s	28	50	33.5	938	+94.0	141.5	796.5	84.9	5.7	790.8	99.2
	6 ♂'s	28	—	33.5	938	+94 0	45.7	892.3	95.4	10.5	881.8	98.8
	6 ♂'s	28	100	67.0	938	+90.5	243.5	694.5	74.0	31.9	662.6	95.4
	6 ♂'s	28	—	67.0	938	+98.6	109.1	828.9	88.4	50.4	778.5	93.9

[1] Figures in this table represent averages per animal.
[2] Corrected for the amount excreted on a thiamin-deficient ration.

thiamin; (C) 6 animals were given 100 mg. of this yeast daily which supplied 67 γ thiamin and 6 were administered 67 γ pure thiamin daily. Such a procedure made it possible to evaluate the absorbability and utilization of thiamin in the brewers' yeast by a comparison with results secured on the pure vitamin. It is apparent from Table I that the thiamin on the 50 mg. daily dose of this yeast, during the maintenance period of 14 days was as well absorbed and utilized as on the pure vitamin. On the 100 mg. daily dose of yeast the utilization of thiamin was the same as on the pure vitamin but the absorption was 12% less on the yeast thiamin as on the pure synthetic vitamin. During the second metabolism growth period the thiamin in the yeast was as well utilized

as on the pure vitamin but here again the absorption was 6 to 11% less in the thiamin derived from the yeast as in the synthetic vitamin. After the completion of the balance studies these 4 groups of animals were killed, and the thiamin content of all the tissues as well as the thymus and adrenals was determined. The pituitary and thyroids did not yield enough thiamin from groups of 6 animals for analysis. From Table II

TABLE II

The Influence of Administration for 42 Days of Thiamin in Brewers' Yeast (B) Versus Pure Crystalline Thiamin on the Concentration of This Vitamin in the Body Tissues of the Albino Rat

Tissue	Group A 33.5 γ thiamin daily in brewers' yeast		Group B 67.0 γ thiamin daily in brewers' yeast		Group C 33.5 γ pure thiamin daily		Group D 67.0 γ pure thiamin daily	
		Total in entire tissue		Total in entire tissue		Total in entire tissue		Total in entire tissue
	γ/g.	γ	γ/g.	γ	γ/g.	γ	γ/g.	γ
Liver	9.66	119.78	9.17	119.21	8.83	108.00	9.50	123.60
Kidney	5.12	10.30	8.04	18.00	4.95	10.90	5.07	11.67
Spleen	2.24	2.40	5.47	5.20	3.10	3.20	3.29	3.07
Heart	9.17	8.67	8.60	8.20	7.94	7.73	7.25	7.40
Stomach	5.29	6.66	5.80	7.20	6.72	7.47	5.76	7.73
Brain	7.95	8.67	5.37	8.00	7.37	8.26	8.00	11.10
Pancreas	2.52	2.77	5.61	5.73	4.37	5.20	4.71	5.60
Lungs	3.31	4.93	3.45	5.73	3.47	4.93	3.82	5.73
Small intestines	2.17	6.87	4.64	10.67	2.23	8.33	3.93	12.67
Large intestines	1.72	2.93	4.26	8.40	2.43	4.70	3.45	7.60
Muscle	0.56	28.56	0.75	37.23	0.52	24.18	0.77	38.42
Testes	11.24	18.33	13.23	22.00	12.70	20.67	13.00	22.50
Thymus	4.43	1.46	5.63	1.80	4.20	1.66	6.45	2.40
Adrenals	9.64	0.27	4.96	0.18	4.70	0.16	4.82	0.16
Total in all tissues		222.60		247.55		215.39		259.65

it is quite clear that the thiamin in brewers' yeast B was just as efficiently utilized for storage of body tissues as the pure vitamin, as evidenced by concentration per gram of each tissue and from the total thiamin content of all tissues. The small differences found are characteristic of what is found among individual animals.

Yeast C

In this series there were 24 males which were placed on experiments when 34 to 43 days of age. The initial weights of the animals ranged from 100 to 182 g. each, which afforded an excellent opportunity to study the influence of body weight on efficiency of thiamin utilization in brewers' yeast C of a potency of 160 γ/g. The daily dose of this yeast was 200 mg., providing 32 γ thiamin. The metabolism study was divided into two periods: (1) A preliminary period of 21 days of depletion, allowing 2 to 3 γ thiamin daily, which kept the animals in maintenance and yielded urinary and fecal thiamin excretions comparable to figures obtained after a severe depletion period of 7 days. (2) A balance study of 21 days, permitting continuous growth by allowing the feed *ad libitum*. It was found that as much as 82% variation in initial and 33% variation in gains of body weight during the metabolism period of balance studies resulted in no noteworthy differences in absorption and utilization of thiamin in brewers' yeast C. The thiamin in this yeast was well absorbed (88.8%) and utilized (97.8%). To conserve space, however, all detailed data are deleted.

Summary

Thiamin balance studies were conducted on three brands of brewers' yeasts which had a potency of 440, 670, 160 γ/g. respectively. The daily dose of yeast supplied 22 to 88 γ thiamin. The results showed excellent utilization of thiamin in these yeasts, ranging from 93 to 100%.

A comparison was made between the absorption and utilization of thiamin in brewers' yeast and that of pure crystalline thiamin. There was no difference in efficiency of utilization but there was 6 to 11% poorer absorption of thiamin in one type of brewers' yeast studied compared with an equivalent intake of pure thiamin.

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On the Mechanism of Enzyme Action. Part 23

Structure and Action of Rubrofusarin from Fusarium graminearum Schwabe (Fgra.) (*Gibberella saubinettii*)

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Introduction

In the course of investigations concerned with the enzyme system of Fusarium oxysporum (Fox.), the observation was made in 1937 (1) that a pigment is deposited in the mycelium of this fungus which serves as a suitable and natural indicator for pH changes occurring within the cell. Judging from this property, it was assumed at that time that this pigment might be related to that which was isolated from a member of the Fusarium culmorum family in the same year (2). The function of these types of compounds in the cell had not been systematically investigated and were, therefore, generally regarded as waste products.

Uncorrelated observations concerning the nature and possible action of the cause of the photosensibilizing effect of a fluorescing dye, *i.e.*, hypericin (3) on microorganisms have been made (4), and, in a recent paper (5) it was also indicated that phoenicin, the red pigment present in Penicillium phoenicium, is capable of exerting an influence on the respiration of Bacillus pyocyaneus. Our interest was, therefore, directed towards a possible influence of Fusaria pigments upon activities of the highly developed dehydrogenating system of this fungus. Efforts were

[1] Condensed from a part of the dissertation submitted by R. P. M. to the Graduate Faculty of Fordham University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The results given in this paper were presented before the Division of Biochemistry at the meeting of the American Chemical Society, Pittsburgh, Pennsylvania, 1943.

[2] Communication No. 33.

consequently made to isolate and identify such compounds present in Fox., Fusarium lycopersici (Flyco.) and Fgra.

Growth experiments upon Raulin-Thom and Czapek-Dox nutrient media, covering a pH range from 2 to 8, indicated that the maximum color formation of Fox., which was comparatively slight, occurred when using the first of these media at a pH of 8. After three weeks growth in a sterilincubator, and extraction of the mycelia with numerous organic solvents, ethanol was found to be the only solvent capable of removing pigment from the mats. Because of the extremely small amount of this material present, however, attempts to isolate the coloring matter from the solution proved futile.

Flyco., the second organism investigated, was found to produce a purple-red mycelium of maximum color intensity at pH 6 on a Raulin-Thom nutrient medium. Of the various solvents employed, chloroform proved to be the best extracting agent of the coloring matter present in the mats. Since the solution appeared to contain several substances, chromatographic adsorption was tried as a means of effecting their separation and purification. It was found that a column of alumina served best as an adsorbent for the mixture. At the end of the development with chloroform a light brick red zone was visible at the top of the column, followed by a thinner light orange zone and a broad purple zone. Only in the case of the first band was any material able to be isolated from the adsorbent. The band was cut out; the alumina dissolved in dilute hydrochloric acid, and the solution neutralized with sodium hydroxide whereupon a flocculent amorphous purple precipitate formed. This precipitate was repeatedly washed with water, centrifuged, and then dried to constant weight in vacuo at 110°. The compound did not melt below 360° (cor.). Analyses for carbon and hydrogen showed varying percentages of these elements, and attempts to obtain a sample giving a constant analysis were unsuccessful. In the presence of sunlight the compound was observed to lose its purple color and become white. Since only 7 mg. of this impure labile material could be obtained after extracting more than 600 g. of dry mycelia, further structural and enzymatic investigations with the pigments obtainable from Flyco. were abandoned. The compound, however, is probably responsible for the different colors found in the mycelia when Flyco. is grown at different pH, since the addition of hydrochloric acid causes this pigment to become pink in color and ammonium hydroxide reconverts it to the purple colored compound.

An attempt was therefore made to obtain a working quantity of the pigments present in Fgra. for further studies. The first of these pigments, rubrofusarin, was isolated in greatest amount when the organism was grown upon the Raulin-Thom nutrient medium at pH 8 for three weeks. The marked coloration of the mats, which is observable within a week when the sterilincubator is employed, does not become evident until three or four weeks have passed if Erlenmeyer flasks are used.

Starting with an iso-propyl alcohol solution of the pigment (6), its effect upon the rate of dehydrogenation by unpigmented Fusarium lini

Bolley (FlB.) was investigated and an *inhibition* amounting to about 3%[3] of the rate could be measured despite an increase of approximately 40% of the final mat weight.

At this point, however, there was no indication yet on hand to correlate the structure of the pigment and its action. The compound was therefore isolated and an attempt was made to elucidate its structure.

Experimental[4]

The technique applied for growing and collecting mycelia and the cultures employed were the same as described heretofore (1, 6, 7).

The following procedure was found best suited to obtain the pigment. The dried and finely ground mycelia were first extracted in a large Soxhlet for two hours with petroleum ether (b.p. 40–60°) to remove most of the fat (8) present. A fresh portion of solvent was then employed to remove the pigment. This second extraction was run at an average of ten to eleven days without changing the solvent. Most of the petroleum ether was then distilled off, and the crude red crystals of rubrofusarin centrifuged from the remainder of the solution.

It was found possible to purify the crude compound by chromatographic adsorption. A petroleum ether solution of the pigment was adsorbed upon alumina, (Merck's Reagent, standardized according to Brockmann) and a single orange colored zone formed at the top of the column. This band, however, was retained too strongly by the alumina and could not be eluted although numerous organic solvents in various proportions were tried. A less active adsorbent consisting of calcium carbonate (Merck) and Hyflo Super Cel (F.A. 501, Johns-Manville) (1:1) was therefore employed. The orange band which formed near the top was successfully eluted with a mixture of petroleum ether and ethanol (2:1). The solvent was evaporated and the orange-red colored needles, after drying in vacuo at 110°, of m.p. 209–210° (cor.) gave satisfactory analytical data.

$C_{15}H_{12}O_5$.	Calculated.	C 66.15,	H 4.44,	$-OCH_3$ 11.40.
	Found.	66.36,	4.52,	11.05.

The second pigment, aurofusarin, was obtained in crude form by employing the same general procedure used for rubrofusarin except that the pH of the nutrient medium was 4, and after removal of the fat with petroleum ether, chloroform was used as the second extracting solvent. The crude form of the compound, which is

[3] Cf. footnote No. 5.

[4] Unless otherwise noted, the melting points given herein are uncorrected.

almost black in color, became lighter when repeatedly washed with cold chloroform and gave the following analysis:

$C_{30}H_{20}O_{12} + H_2O$. Calculated. C 60.99, H 3.76.
Found. 60.16, 4.38.

After numerous such washings, the compound was dissolved in chloroform and adsorbed upon a column of alumina upon which it formed a single deep purple colored band which was eluted with a mixture of chloroform and acetic acid (5:1). The percolate was bright red in color, and upon concentration and centrifuging the solution, a red amorphous powder was obtained which did not melt below 360° (cor.) although darkening began at about 250° (cor.). Microanalysis indicated that the compound remained impure when this method was employed. An attempt to further purify the compound in a manner similar to that employed by Raistrick and co-workers (2), *i.e.*, by boiling with acetic acid and charcoal, was made. A light yellow compound which could not be analyzed because of its hygroscopicity was obtained when the treatment was prolonged, indicating that this method of purification if extended for several hours results in some alteration of the substance. For the time being, no further attempts were made to purify the compound.*

The results of a series of growth experiments designed to obtain pigments from Fusaria are shown in Table I.

Investigation of the Structure of Rubrofusarin

Although rubrofusarin is believed to be a monomethyl ether of a methyl trihydroxyxanthone, the position of the groups in the complex molecule is not certain. This is partially due to the small quantity of pigment obtainable, and the difficulty encountered in attempting to degrade the compound by ring cleavage. While the presence of certain groups may be indicated by chemical tests, the positions of such constituents are not necessarily shown by this means. However, by comparing the absorption spectra of synthesized compounds of known similar structure with the spectrum of the unknown compound, one can often eliminate certain structural possibilities. It was found necessary, there-

* A pigment obtained from a growing culture of Fusarium solani D_2 purple (FsD.), which diffuses into the surrounding medium was also isolated. The organism was grown on the usual Czapek-Dox medium as well as on one to which traces of Zn and Mn were added. After 2–3 weeks of growth the dried mycelia were ground and extracted with 0.5% NH_4OH solution. Upon acidification and concentration in vacuo the crude pigment was separated from the inorganic constituents by extraction with toluene. An attempt was made to establish the absorption spectra of an alcoholic solution of this pigment but no measurable maxima could be observed in the range of 700–1500 f. In this respect the pigment exhibits the same behavior as aurofusarin. The pigment obtained from FsD. is purple colored in alkaline, and orange colored in acid medium and distinguishes itself from aurofusarin through its remarkable solubility in water.

fore, to prepare a number of xanthones containing hydroxy, methoxy and methyl groups in various positions of the nucleus and to determine

TABLE I

Production of Pigments and Fats from Fusaria

Fusaria	Initial pH	Final pH	Incubation period in days	Weight of mycelia g.	Weight of crude pigment g.	Weight of crude fat g.
Fox.	8.0	7.3	21	380.4	0.002	
	8.0	6.5	24	405.6	0.003	
Flyco.	6.3	6.6	21	305.2	0.004	
	6.5	6.8	21	300.4	0.003	
Fgra.	4.0	2.8	21	310.0	26.900	32.0
	8.0	7.2	21	405.5	0.231	38.2
	8.0	6.3	23	401.2	0.220	39.0
	8.0	4.3	25	344.6	0.188	31.1
	8.0	5.4	26	350.5	0.186	32.4
	8.0	4.4	32	287.9	0.171	29.6

TABLE II

List of Xanthones Used

Compound	Melting Point Found	Analysis* Calculated C %	Calculated H %	Found C %	Found H %
Xanthone........	174–176°	79.59	4.08	79.61	4.13
1-Hydroxy-xanthone..	147–148°	73.58	3.76	73.79	3.94
1-Methoxy-xanthone..	138–139°	74.33	4.42	74.47	4.22
1-Hydroxy-3-methyl-xanthone...........	140–141°	74.33	4.42	74.43	4.27
1-Hydroxy-6-methyl-xanthone............	172–174°	74.33	4.42	74.41	4.36
1,6-Dihydroxy-xanthone............	242–243°	68.42	3.51	68.76	3.73
1,8-Dihydroxy-xanthone............	187–189°	68.42	3.51	68.26	3.58
3-Hydroxy-xanthone...	243–245°	73.58	3.76	73.26	3.92
4-Hydroxy-xanthone...	240–242°	73.58	3.76	73.46	3.94

* All microanalyses were carried out by J. J. Alicino of Fordham University and The Squibb Institute.

their absorption spectra. The synthesis of ravenelin, which is isomeric, but not identical with rubrofusarin, was also undertaken.

The xanthones listed in Table II were prepared by methods which are available in the literature.

Synthesis of 2-Hydroxy-6-methoxy-benzonitrile

2-Hydroxy-6-methoxy-benzaldoxime. A mixture of 1.4 g. of 2-hydroxy-6-methoxy-benzaldehyde (9), 1.1 g. of hydroxylamine hydrochloride, 4 ml. of pyridine and 4 ml. of ethanol was refluxed for two hours on the steam bath. The solvents were removed by evaporation in a current of air. The residue was triturated with 5 ml. of cold water and let stand in the ice box over night. The white needles of the oxime which separated were crystallized from an ethanol-water mixture; yield 1 g.; m.p. 60–62° (cor.).

$C_8H_9O_3N$. Calculated. N 8.38.
Found. N 7.96.

2-Hydroxy-6-methoxy-benzonitrile. A mixture of 1 g. of the benzaldoxime and 10 ml. of acetic anhydride was refluxed for three hours. The solution was poured into cold water and the reddish oil which separated was extracted with ether, dried over anhydrous sodium sulfate and the ether evaporated in vacuo. The residual oil was dissolved in about 200 ml. of 10% sodium hydroxide and heated under reflux for fifteen minutes at 55°. After cooling, the solution was acidified with hydrochloric acid and the oil which separated was extracted with ether. The ethereal solution was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The compound was crystallized from water; yield 0.4 g.; m.p. 136–138° (cor.).

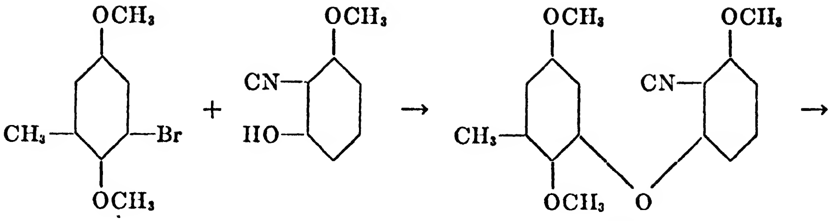
$C_8H_7O_2N$. Calculated. N 9.40.
Found. N 9.06.

Synthesis of Ravenelin (3-Methyl-1,4,8-trihydroxy-xanthone)

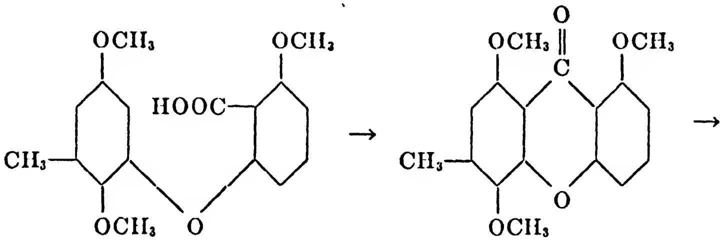
2-Cyano-3′-methyl-3,2′,5′-trimethoxydiphenylether (III). A mixture of 0.2 g. of potassium dissolved in 2 ml. of absolute ethanol, 0.4 g. of 2-hydroxy-6-methoxy-benzonitrile (II), 0.7 g. of 3-bromo-2,5-dimethoxytoluene (I) (10) and 0.1 g. of copper bronze was refluxed at 200° for eight hours. The residue was extracted with ether, dried over anhydrous sodium sulfate and evaporated in vacuo; yield 80 mg.

2-(2′,5′-Dimethoxy-m-tolyloxy)-6-methoxybenzoic acid (IV). A mixture of 80 mg. of the impure cyanodiphenylether (III) and 8 g. of barium hydroxide dissolved in 8 ml. of water was refluxed for twelve hours. The cooled solution was acidified with hydrochloric acid, centrifuged, and the precipitate crystallized from alcohol; yield 30 mg.; m.p. 189–192° (cor.).

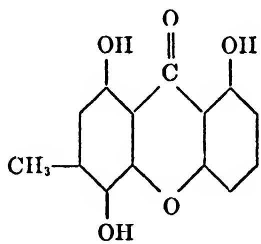
$C_{17}H_{18}O_6$. Calculated. C 64.12, H 5.70.
Found. C 64.46, H 5.92.



I II III



IV V



VI

Synthesis of Ravenelin

3-Methyl-1,4,8-trimethoxyxanthone (*V*). A mixture of 30 mg. of the carboxydiphenylether (IV) and 7 ml. of phosphorus oxychloride was boiled under reflux for three minutes. The solution was made alkaline with sodium hydroxide and centrifuged. The precipitate was purified by sublimation in vacuo; yield 22 mg.; m.p. 179–180° (cor.).

$C_{17}H_{16}O_5$.	Calculated.	C 67.97, H 5.37.
	Found.	C 67.92, H 5.45.

3-Methyl-1,4,8-trihydroxyxanthone (*VI*). Twenty-two milligrams of the trimethoxyxanthone (V) was demethylated by heating with 2 ml. of hydriodic acid in a Zeisel apparatus at 95–100° for five hours. The

cold mixture was poured into water, centrifuged and triturated with sodium thiosulfate solution, washed with water and dried. The compound was purified by sublimation in vacuo; yield 19 mg.; m.p. 267–269° (cor.). A mixed melting point with *ravenelin* obtained from *Helminthosporium ravenelii* Curtis gave no depression.

$C_{14}H_{10}O_6$. Calculated. C 65.10, H 3.91.
Found. C 65.21, H 4.13.

I. *Absorption Spectra of the Xanthones (Figs. 1–9)*

Spectral Range 800–1000 f. In the far ultraviolet region the xanthones are found to have characteristic maxima at 825–890 f and minima at

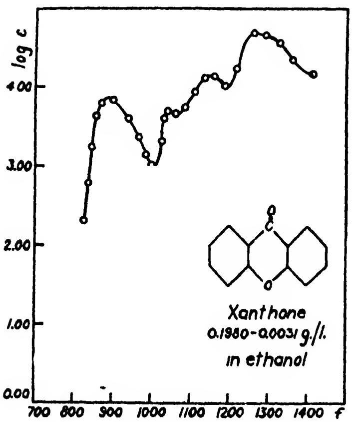


FIG. 1

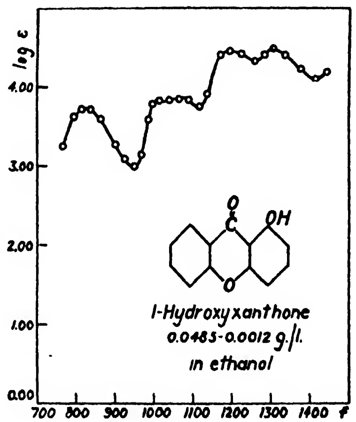


FIG. 2

940–1000 f, except in the case of 3-hydroxyxanthone whose lower maximum is found to be less sharp at the slightly higher frequency of 975 with a corresponding increase of the minimum to 1075 f. These characteristic absorptions are also present in the dihydroxyxanthones, but the difference in extinction between them in the case of the 1,6-dihydroxy compound is less marked while the 1,8-dihydroxyxanthone is found to possess an additional minimum at 800 f.

Spectral Range 1000–1200 f. The addition of various groups to the xanthone nucleus is evidenced mainly in the region of 1000–1200 f. The 1-hydroxyxanthone causes an almost complete elimination of absorption in this range and methylation of the compound diminishes this characteristic still more. The 3-hydroxy compound, while not

showing as much influence in this respect, does exert a similar effect. In the 4-hydroxyxanthones, the two maxima, corresponding to those present in xanthone at 1040 f and 1150 f, merge so that this hydroxy compound has only one maximum in this proximity. A slight decrease

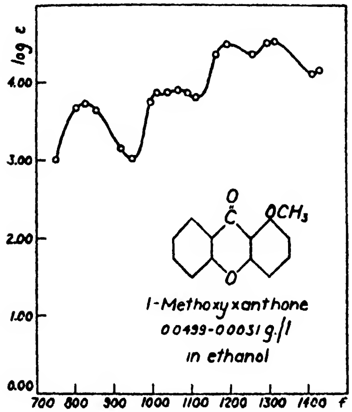


FIG. 3

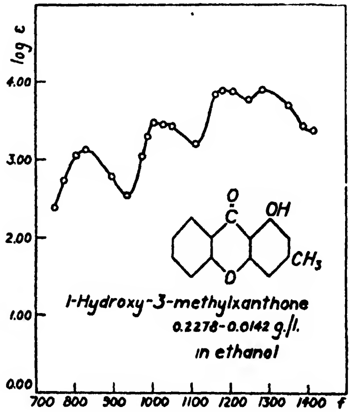


FIG. 4

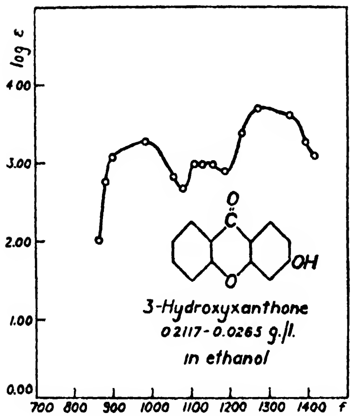


FIG. 5

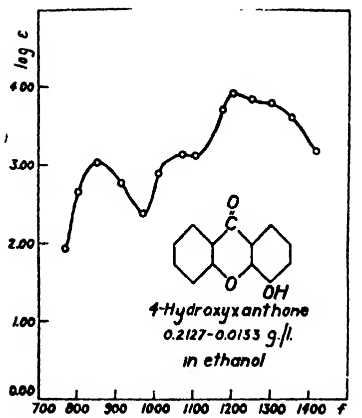


FIG. 6

in the sharpness of the maximum is accomplished by methylation of the hydroxy group. The addition of a methyl group in position three of the xanthone nucleus, as shown by 1-hydroxy-3-methylxanthone, results in decreasing the relative values of log ε of the two maxima and one minimum corresponding to those present in xanthone at 1045 f, 1154 f,

and 1065 f, respectively. In the case of 1,6-dihydroxyxanthone, the 6-hydroxy group causes the two maxima to be in inverse relation to one another. The maximum corresponding to that present at 1045 f in xanthone is now the larger and more marked one while the second maximum corresponding to that at 1150 f in xanthone is now of a secondary nature.

In the case of 1,8-dihydroxyxanthone, this second maximum is almost entirely eliminated by the additional hydroxy group in position eight. The only evidence of it is a slight dip in the slope at 1175 f.

Spectral Range 1200–1400 f. In the near ultraviolet the almost imperceptible minimum detectable in xanthone at approximately 1275 f

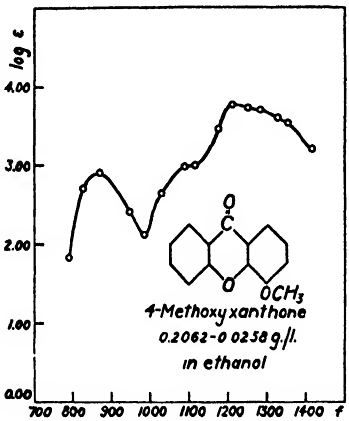


FIG. 7

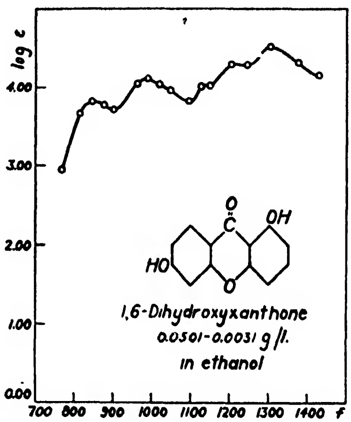


FIG. 8

is accentuated by the addition of various groups, but especially so where a hydroxy or methoxy group is attached to the number one position.

Absorption Spectrum of Ravenelin (Fig. 10)

With the spectra of the aforementioned xanthones available, an attempt was made to see if the structure of this pigment could be established from such information alone. The characteristic maxima and minima present in the xanthones at approximately 825–890 f and 940–1000 f respectively are present at a slightly higher frequency, but without the alteration in the slope and size of the maxima noticeable in 3-hydroxyxanthone; thus the possibility of a hydroxy group in position three may be eliminated.

The slight dip in the curve at approximately 1175 f in the case of

1,8-dihydroxyxanthone is present in the ravenelin spectrum. The accentuated minimum at 1250 f, characteristic of a 1-hydroxy group, together with the additional minimum at approximately 800 f, appear also. These resemblances tend to indicate that the pigment is a 1,8-dihydroxyxanthone.

A comparison of the absorption spectra of 1,6-dihydroxyxanthone and ravenelin fails to show any similarities, except for the previously discussed minima at 1250 f, so that there is no basis for assuming a hydroxy group in position six of the xanthone molecule.

The trend of 1-hydroxy-3-methylxanthone to decrease the relative values of log ϵ for the maxima and minimum between 1015–1150 f when

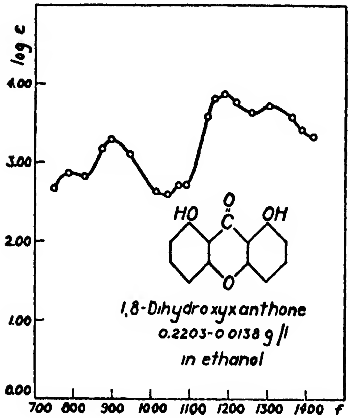


FIG. 9

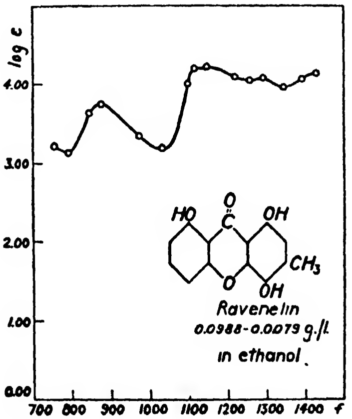


FIG. 10

collated with xanthone is apparent in the spectrum of ravenelin; consequently this xanthone has a methyl group in position three of the molecule.

Except for the fusion of the two maxima in the range 1050–1150 f exhibited by 4-hydroxyxanthone, no conclusion may be drawn from a comparison of ravenelin with this xanthone. Upon a comparative study of the xanthones and ravenelin, it appears that a 1,8-dihydroxy-3-methylxanthone structure of ravenelin is likely with the position of the third hydroxy group being unsettled.

Absorption Spectrum of Nor-rubrofusarin (Fig. 11)

Since nor-rubrofusarin is isomeric although not identical with ravenelin, their absorption spectra were compared as the first step in an

attempt to determine the structure of methylated nor-rubrofusarin (rubrofusarin) from purely spectroscopic findings. The similarity between the spectra of the two compounds is most noticeable in the range 1200–1400 f, indicating the presence of a hydroxy group in position one of nor-rubrofusarin. The slight maxima appearing in the spectrum of 1,8-dihydroxyxanthone at 1075 f and at 900 f in nor-rubrofusarin, while indicating the absence of a hydroxy group in position four of the xanthone nucleus, suggest that the demethylated compound is a 1,8-dihydroxy-xanthone. Since there is no resemblance to the spectra of 1-hydroxy-3-methyl- or 3-hydroxy-xanthones in the region of approxi-

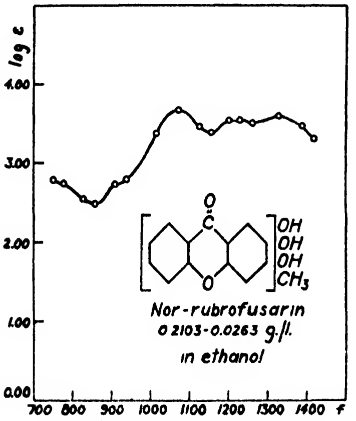


FIG. 11

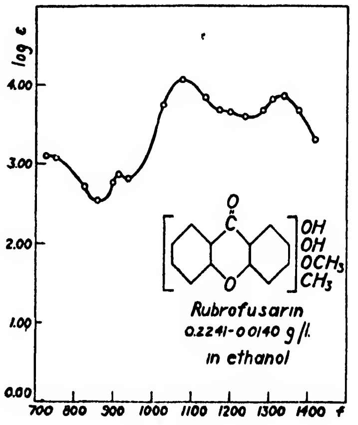


FIG. 12

mately 1050 f, the probability of either a methyl or hydroxy group in position three is not very great.

Since positions three and six as well as four and five are equivalent in the xanthone nucleus, it seems likely that the methyl and third hydroxy groups are in positions two and seven. A possible structure for nor-rubrofusarin therefore would be 1,2,8-trihydroxy-7-methyl-xanthone.

Absorption Spectrum of Rubrofusarin (Fig. 12)

From a comparison of the hydroxy and methoxyxanthones, it is evident that the elimination of the sharpness of a maximum appears to be a characteristic alteration caused by methylating the former compounds. In the spectrum of rubrofusarin this phenomenon is apparent

in the region of 1150–1250 f, the range indicating the presence of a hydroxy group in position one. Rubrofusarin, therefore, may possess either a 2,8-dihydroxy-1-methoxy-7-methyl- or 2,3-dihydroxy-8-methoxy-7-methylxanthone structure.

Discussion

The living cell is acknowledged to be a complex system capable of forming a wide variety of organizers and substances, yet many of its metabolic products deposited within the cell have been designated as waste materials, and a study of their function in the complete "spectrum" of the cell has been largely neglected. Tappeiner (3, 11), in reference to the photodynamic action of such fluorescing compounds as hypericin has stated that: ". . . Zusatz von fluoreszierender Substanz in geringer Menge hingegen hebt das spezifische Wirkungsvermögen (of enzymes) rasch und dauernd auf." On the other hand Friedheim (5) believes to have observed a 200–300% increase in the respiration of Bacillus pyocyaneus, upon the addition of phoenicin. Thus there are recorded two specific instances of "waste" material exhibiting, not only diversified, but also completely contrary functions; the one manifesting an irreversible effect upon the enzyme system concerned and the other an intensification of the respiratory action.

According to Schöpf (12) the formation of natural substances in the living cell may be of three types. The cell may have an enzymatic system designed for the highly specific synthesis of a certain substance or, the processes of general application performed by enzymes, *e.g.*, hydrogenations, dehydrogenations, decarboxylations, etc. Finally there are those which take place without the action of enzymes and are characterized by the formation in the growth of the cell of reactive organic substances. These compounds upon contact in the cell yield isolable products which are, in fact, chance products or intermediates if their further conversion within the cell proceeds slower than the aforementioned synthesis.

The pigments present in microorganisms may be considered to result from the first or last of these three methods of formation, but their relation, if any, to the second type is not always clear. In the case of one of the pigments obtainable from Fgra., however, the compound had a marked effect upon the activity of a dehydrogenating enzyme system present.

This mode of action of rubrofusarin (cf. page 420) and the related xanthones, which contrary to the former, according to the actual struc-

ture, *increase* the rate of dehydrogenation from 5.33% to 12.84%,[5] indicates that the xanthones have a dual but interdependent effect upon the dehydrogenating enzyme systems present in Fusaria. Thus, in a manner analogous to nicotinic acid, they may enhance but may also inhibit the synthesis and/or the action of dehydrogenating enzymes by the microorganism, according to the structure of the xanthone employed while simultaneously promoting or retarding the growth of the mold.

It is evident, therefore, that substances which have heretofore been regarded as insignificant or merely "waste" products should be included in considerations when the mechanisms involved in the enzymatic activities of molds are being investigated.

Acknowledgments

The culture of *Helminthosporium ravenelii* Curtis was obtained through the courtesy of Prof. H. Raistrick, F.R.S., School of Hygiene and Tropical Medicine, London, England.

The investigation was carried out with the material assistance of a grant from the Rockefeller Foundation.

Summary

1. Two pigments, rubrofusarin and aurofusarin, were isolated from Fgra., and the former was purified by chromatographic adsorption.

2. Since rubrofusarin is a member of the xanthone family, additional knowledge of the structure of the compound was sought by synthesis of related xanthones and comparative studies of their absorption spectra.

3. A possible structure of this pigment based upon spectroscopic studies is either 2,8-dihydroxy-1-methoxy-7-methyl- or 2,3-dihydroxy-8-methoxy-7-methylxanthone.

4. The structure of the xanthone revenelin was confirmed by synthesis, and a spectroscopic analysis of its molecular constitution undertaken.

5. Xanthones may enhance or inhibit the synthesis and/or the action of dehydrogenating enzymes by Fusaria.

6. The role of these "waste" materials in the metabolism of Fusaria cells is discussed.

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[5] These values, as well as that on page 421, are representative for three series of experiments, each run in duplicate.

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Studies on the Colorimetric Determination of Cystine[1]

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Introduction

In the progress of an investigation reported in the accompanying paper (1) we desired to have a sensitive yet specific method for the determination of the sum of cysteine and cystine in the presence of glutathione. The Sullivan method using sodium β-naphthoquinone-4-sulfonate was chosen as the one most likely to be suitable for this purpose because of its reported specificity (2). In our use of the method some observations of possible general interest were made and they are reported in this paper.

Experimental

The procedure as used by Sullivan and Hess (3) was adapted for use in the Evelyn photoelectric colorimeter (4) with a color filter having a maximum transmission at 524 mμ. Cysteine oxidized at neutrality with air in the presence of cupric ion (3×10^{-6} M $CuSO_4$) and cystine gave the same color value in the method. The plot of cysteine or cystine concentration against the log of the galvanometer reading given by the sample, corrected for the blank, was linear up to 0.4 mg. cystine and deviated only slightly from linearity up to twice this amount (Fig. 1).

Although it has been reported frequently that the Sullivan method responds to cysteine and not to glutathione, it was considered advisable to check the effect of glutathione and of several other compounds under the conditions used. A convenient method of doing so was to follow the light absorption of the color produced by the compounds with the dye under the conditions of the method. The only data we could find on the

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absorption characteristics of the cystine-dye color was that given by Bushill, Lampitt, and Baker (5).

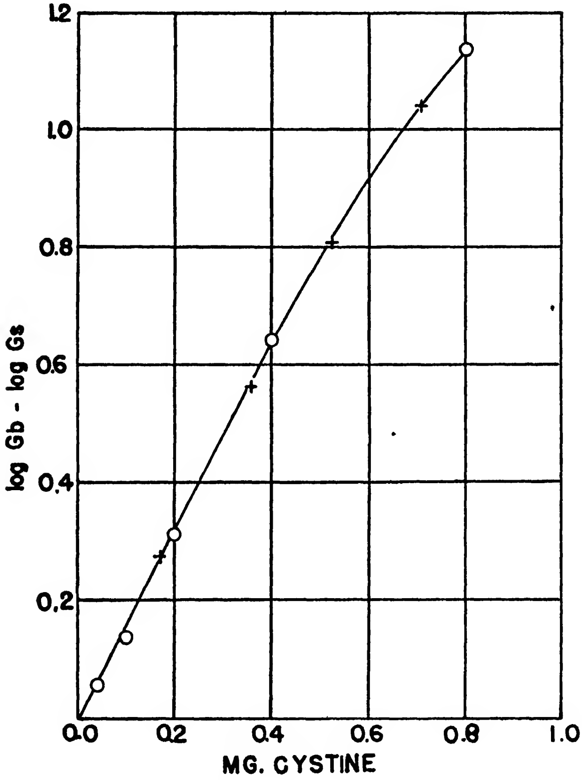

FIG. 1

Relation between Color Production and Cystine Concentration in the Sodium β-Naphthoquinone-4-Sulfonate Method for the Determination of Cystine

A neutral 5 ml. sample of cystine (O) or cysteine (X) oxidized with air in the presence of cupric ion was used. Two ml. of 5 per cent NaCN in 1.0 N NaOH were added to the neutral sample to be analyzed. At the end of ten minutes, 2.0 ml. of aqueous 1.0 per cent sodium β-naphthoquinone-4-sulfonate were added and after 15 seconds, 5.0 ml. of 10 per cent Na_2SO_3 in 0.5 N NaOH were added. After 30 minutes in the dark at room temperature (20–25°), 2.0 ml. of 5 N NaOH and 1.0 ml. of 2 per cent $Na_2S_2O_4$ in 0.5 N NaOH were added. The tube was shaken after each addition. After 5 minutes, the galvanometer reading of each of the samples was taken in the Evelyn photoelectric colorimeter and used to determine the curve. A blank containing distilled water in place of the cystine was run at the same time.

We have measured the light absorption of the dye by means of a Beckman spectrophotometer (6) and have determined the effect of several compounds on it. Fig. 2 shows the curve for the light transmission of the dye alone under the conditions used in the determination. As shown in the same figure, the color produced by the reaction of the dye with cystine gave a maximum absorption at 500 mμ and a minimum at 440 mμ when corrected for the absorption of the dye carried through in a blank and used as the zero settings, *i.e.*, 100 per cent transmission for the spectrophotometer at each wave length setting. The optical density (log 100 − log per cent transmission) of a sample of the cystine-dye color run against distilled water as the blank was equal to the sum of the optical density of the cystine-dye color run against the dye as a blank plus the optical density of the dye itself run against distilled water as a blank. Cystine was run at a concentration of 1.67×10^{-4} *M* over the range of 440 mμ to 600 mμ. Below 440 mμ the light transmission of the dye and the cystine-dye color became negligible under the conditions tested.

Glutathione, glutamic acid, and glycine apparently each reacted with the dye under the conditions of the cystine test but the absorption in the region of maximum absorption of the cystine-dye color was not great although it was not negligible when the concentration of these materials was much greater than that of the cystine being determined (Fig. 3).

It was noted in the course of some other work that thioglycolic acid interfered in the determination of cystine by the above method. For this reason the spectrum of the mixture of thioglycolic acid and dye was determined. It can be seen (Fig. 2) that thioglycolic acid, when present in sufficient amounts, gave almost the same color as cystine. The intensity of color produced was proportional to the concentration of thioglycolic acid over the range of 0.0 to 5.3 mg. and was approximately one-fifteenth of that given by an equal weight of cystine. Above a level of 5.3 mg. of thioglycolic acid, which is equivalent to 64 per cent of the dye present, the relation of color produced to concentration of thioglycolic acid became erratic; at higher levels, where the molar concentration of thioglycolic acid exceeded that of the dye, the color intensity decreased.

Although glutathione did not give much interfering color by itself in the cystine method, when reduced glutathione was added to cystine or cysteine and the mixture was oxidized by air, the color obtained was much greater than that obtained from cystine or oxidized cysteine alone. The increase was dependent on the ratio of glutathione to cysteine. Mixtures containing several different ratios of glutathione to cysteine were oxidized with air and then analyzed for cystine. The results are shown in Table I. It is apparent that more cystine was found than was added. A possible explanation of the finding is that oxidation of the mixtures results in the formation of a mixed disulfide of cysteine and glutathione.

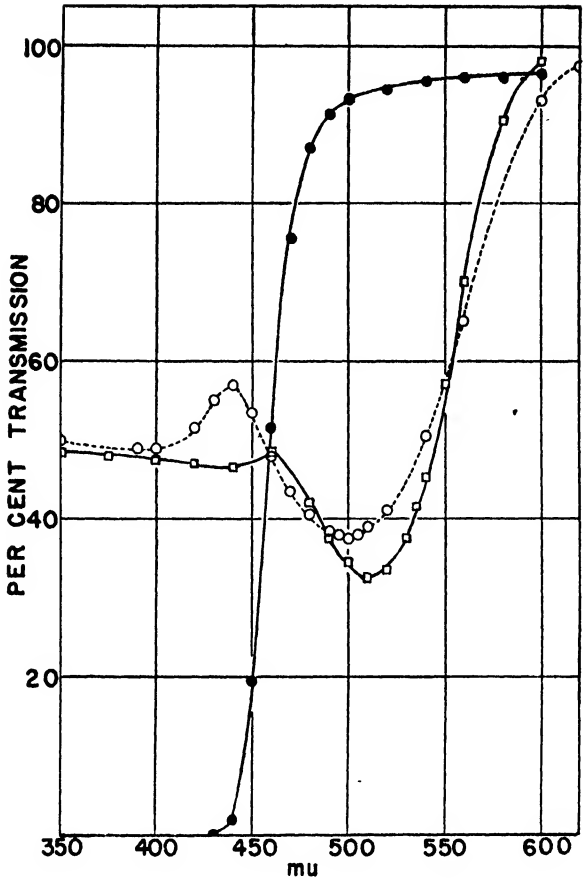

FIG. 2

Light Absorption of Sodium β-Naphthoquinone-4-Sulfonate and of the Color Produced by Cystine and Thioglycolic Acid on Reaction with It

The sample was carried through the same procedure as used in the regular colorimetric test. The instrument was set to give 100 per cent transmission with the blank at each wave length and then balanced again with the sample in place. The plot relates wave length and per cent transmission of the sample. Curve ○ represents 3.3×10^{-4} *M* cystine; curve □ represents 6.0×10^{-3} *M* thioglycolic acid; curve ● gives the light absorption of the dye itself when the instrument was set to give 100 per cent transmission with distilled water as the standardizing medium. The concentrations of cystine and thioglycolic acid are expressed on the basis of the 5 ml. sample used in the colorimetric determination. The thioglycolic acid was purified by distillation.

The mixed disulfide upon reduction with NaCN in the determination may yield more than 50 per cent of its cysteine as the reactive free sulfhy-

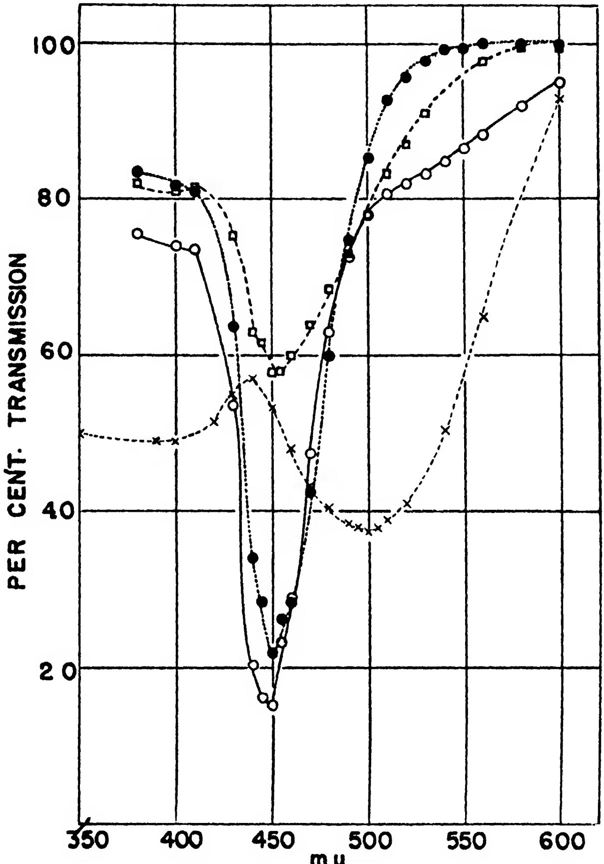

FIG. 3

Light Absorption of the Color Produced by Glutathione, Glutamic Acid, Glycine, and Cystine on Reaction with Sodium β-Naphthoquinone-4-Sulfonate

The sample was carried through the usual colorimetric procedure. The plot relates wave length and per cent transmission of the sample measured with the instrument set at 100 per cent transmission at each wave length for a reagent blank. The concentrations of materials used were as follows: glutathione (○) 1.0×10^{-2} M; glutamic acid (□) 2.0×10^{-2} M; glycine (●) 2.0×10^{-2} M. For comparison, the plot of wave length and per cent transmission of cystine at a concentration of 3.3×10^{-4} M is given (×).

dryl and a correspondingly greater percentage of its glutathione as the cyano derivative while cystine upon reduction with NaCN yields 50 per cent cysteine and 50 per cent of the unreactive S-cyanocysteine (7). On such a basis one should expect that mixtures of cystine and oxidized glutathione would behave normally in the cystine determination and, as Table I shows, such is actually the case. The fact that mixtures of the two compounds in the reduced form can be correctly analyzed for cysteine was previously demonstrated by Sullivan and Hess (8). Preliminary

TABLE I

Effect of Glutathione on the Determination of Cystine

Sample No.	1	2	3	4	5	6	7	8	9	10	11
GSH present, mg.	1.23	1.53		0.20		0.20		0.92	1.53	0.61	0.31
GSSG present* as mg. GSH					0.20		0.20				
CSH present as mg. CSSC	0.12	0.12	0.14	0.14				0.24	0.24	0.36	0.48
CSSC present†, mg.					0.14	0.16	0.16				
CSSC determined, mg.	0.21	0.22	0.14	0.21	0.13	0.24	0.17	0.38	0.41	0.55	0.60

* The oxidized glutathione was prepared by air oxidation of reduced glutathione.

† The cystine of sample number 5 was prepared by air oxidation of cysteine.

Solutions of reduced glutathione (GSH), oxidized glutathione (GSSG), cysteine hydrochloride (CSH), and cystine (CSSC) in 3 per cent metaphosphoric acid were mixed to give 5.0 ml. of the desired composition. After neutralization and addition of cupric ion (3×10^{-6} *M*), the samples were oxidized and the volume made to 10.0 ml. A 5.0 ml. aliquot was tested for cystine by the regular procedure.

experiments indicate that other sulfhydryl compounds show an interference in the cystine determination similar to that described for glutathione.

Summary

The absorption curve of the color produced by the reaction of cysteine with sodium β-naphthoquinone-4-sulfonate is reported. It is shown that the color produced by the reaction of thioglycolic acid with the same dye is very similar to that produced by cysteine while the colors produced by

glutathione, glutamic acid, and glycine are quite different. It is shown that if mixtures of cysteine and reduced glutathione are oxidized and analyzed for cystine the value comes out too high. A possible explanation is suggested.

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Glutathione Hydrolysis by Guinea Pig Liver[1]

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Introduction

We have recently had occasion to determine the total reducing groups in deproteinized preparations from guinea pig liver. To do so we added metaphosphoric acid, to a final concentration of three per cent, to homogenates or extracts of the liver, centrifuged, and titrated the supernatant extract with iodine. We were surprised to find that the amount of iodine used increased rapidly as the time between the killing of the animal and the addition of the metaphosphate increased. Investigation of the literature revealed that essentially the same finding had been observed before. The increased titration has been explained as due to reduction of oxidized glutathione (1); the liberation of glutathione by protein hydrolysis (2,3) and the hydrolysis of glutathione with the production of sulfhydryl compounds of greater reducing capacity than the glutathione itself (2,4,5). Since the character of the end point changed during the increase in titration from a sharp definite break, that is characteristic of glutathione under such conditions, to one that is less sharp and definite and more characteristic of cysteine, the third possibility seemed most likely. The work described below was carried out to investigate this possibility and if it proved correct to study the process by which it was brought about.

Methods

The liver of normal guinea pigs was used throughout the work except in the comparative studies. The pigs were maintained on a Rockland diet fed *ad libitum*. The animals were killed by cutting their throats and allowing them to bleed as

[1] The authors are indebted to the Buhl Foundation for a research grant in support of this investigation.

Contribution No. 529 from the Department of Chemistry, University of Pittsburgh.

much as they could. The liver was then removed as quickly as possible and used in the test desired. A small portion of the liver was immediately ground with enough metaphosphoric acid to give a 3 per cent solution and it was taken as the zero time sample. The remainder of the liver was homogenized with 2 volumes of $M/15$ phosphate buffer of pH 7.4 and allowed to incubate at room temperature. At suitable intervals, aliquots were removed, deproteinized with one-half volume of 10 per cent metaphosphoric acid and centrifuged. All residues after centrifuging were washed with 3 per cent metaphosphoric acid, and the washings were added to the supernatant extracts of the samples.

Free cysteine plus cystine in the deproteinized extracts was determined by the Sullivan and Hess method as discussed in the accompanying paper (6). A suitable aliquot was neutralized with 0.1 M NaOH, oxidized with air in the presence of 3×10^{-6} M $CuSO_4$ and used in the method.

Iodine titrations were made on the 3 per cent metaphosphoric acid extracts with 0.001 M iodine (0.0013 M in KI). Controls of cysteine, reduced glutathione, and mixtures of these compounds were run. Table I indicates the quantities of 0.001 M iodine that were required to oxidize 5.0 ml. samples of 0.001 M glutathione, of

TABLE I

Titration Value of 5.0 Milliliter Samples of Approximately 0.001 M Glutathione, 0.001 M Cysteine, and Mixtures of the Two

Ratio of GSH to cysteine . .	5:0	4:1	3:2	2:3	1:4	0:5
Ml. 0.001 M iodine required....	5.55	7.39	9.65	11.72	13.09	13.93

The glutathione and cysteine (as the hydrochloride) were dissolved in 3 per cent metaphosphoric acid to give 0.001 M solution. After mixing, the samples were titrated directly with 0.001 M iodine using starch as an indicator. The temperature of the solutions was 23°.

cysteine, and of mixtures of the two in 3 per cent metaphosphoric acid at 23°. All the samples contained the same amount of total sulfhydryl but it was made up of different proportions of cysteine and glutathione. Under the conditions of the titration, 1.0 mg. of cysteine required 13.8 ml. more of 0.001 M iodine than an equivalent amount of glutathione. This value was used to calculate the cysteine increase found in successive deproteinized extracts of liver.

Total cystine determinations were made on the deproteinized extracts after hydrolysis in 20 per cent HCl for 6 hours at 125°. After neutralization the hydrolyzates were carried through the usual colorimetric procedure with corrections for the darkening due to humin formation and loss of cystine during hydrolysis.

Kjeldahl nitrogen determinations on the deproteinized extracts were made by the micro Keyes method (7).

Experimental

Successive deproteinized aliquots of homogenized guinea pig liver showed an increase of both iodine titration and color value in the colori-

metric method up to 40 to 60 minutes incubation. Unless special precautions were taken the two methods did not give the same result for two reasons. Some oxidation of sulfhydryl occurred during a number of the experiments and this made the iodine titration low. The colorimetric value tended to be high at intermediate points due to the interference of glutathione as described in the previous paper (6). However, both values increased together and approached a limiting value. Table II shows successive iodine titrations and cystine determinations on the deproteinized extracts of incubating guinea pig liver.

TABLE II

Free Cystine and Iodine Titration Values of Deproteinized Extracts of Guinea Pig Liver

	Time after removal of liver from animal (minutes)							
	0	10	15	20	30	40	50	60
Animal 1								
Iodine titration*....	12.5		16.1	17.1		21.6		22.6
Colorimetric cystine†...	0.19		0.4	0.58		0.89		1.00
Animal 2								
Iodine titration... .. .	13.3	15.2		19.4		24.1		25.4
Colorimetric cystine....	0.18	0.36		0.71		0.98		1.08
Animal 3								
Iodine titration... .. .	13.1	15.4		21.3		26.3		26.3
Colorimetric cystine....	0.17	0.35		0.74		0.90		0.87
Animal 4								
Iodine titration.... ..	7.0			8.5	10.0	10.5	11.3	11.4
Colorimetric cystine....	0.16			0.53	0.68	0.78	0.8	0.80
Animal 5								
Iodine titration..... .	14.6	17.9		20.6	23.1	25.0		25.6
Colorimetric cystine....	0.31	0.56		0.75	0.93	0.93		0.87

* Expressed as milliliters of 0.001 M iodine per gram of tissue.

† Expressed as milligrams of cystine per gram of tissue.

The Kjeldahl nitrogen and total cystine on the successive samples were the same as, or slightly less than, the same determinations on the zero time sample taken before incubation. Table III gives the results of a representative experiment and indicates that both of these values remained quite constant. These data indicate clearly that proteolysis of

tissue with the formation of glutathione or cysteine cannot be the explanation of the increase in iodine titration. The total cystine of the original sample and of the successive samples was equal to the highest value found for cysteine in the incubated unhydrolyzed samples. This finding shows that the free cysteine increase observed must have been derived from the total cysteine present in the metaphosphoric acid soluble material preformed in the liver before incubation.

TABLE III

Free Cystine, Total Cystine, and Kjeldahl Nitrogen in Deproteinized Extracts of Guinea Pig Liver

Minutes of incubation before deproteinization	0	13	23	33	43
Colorimetric cystine, mg. per g. tissue	0.25	0.38	0.64	0.75	0.83
Total cystine after hydrolysis, mg. per g. tissue	0.94	0.81	0.81	0.88	0.83
Kjeldahl nitrogen, mg. per g. tissue	1.22	1.05	1.07	1.07	

TABLE IV

Production of Cystine from Reduced and Oxidized Glutathione

Minutes incubation	0	10	20	30	60
	5.74 mg. GSH added per g. of tissue				
Colorimetric cystine, mg. per g. tissue	1.52			2.80	2.87
	1.66	2.44	3.07	3.12	
	5.74 mg. GSSG added per g. of tissue				
Colorimetric cystine, mg. per g. tissue	1.58			2.32	2.81
	1.40	1.92	2.06	2.15	

An attempt was made to determine glutamic acid in the tissue extracts by Cohen's method (8) but recovery of glutamic acid added to fresh incubating liver was not quantitative. Apparently some other reaction was affecting the content of this amino acid.

The addition of glutathione to liver homogenate which had reached its maximum cysteine value was followed by a further increase of free cysteine as indicated by the colorimetric test and iodine titration. The addition of oxidized glutathione was also followed by a similar increase in cystine (Table IV) as indicated by the colorimetric test. The data with

oxidized and reduced glutathione are not quantitatively comparable because of the difference in the effect on the cystine determination (6), but they clearly show the same trend. The increase in cystine can be made greater by the addition of larger amounts of glutathione.

Since it has been reported that γ-glutamylcysteine (9) does not give any color in the cystine determination and Harington and Mead (10) have found that it titrated only about 10 per cent high in the iodine titration used by them it seems likely that our findings can be explained only by the formation of cysteine or cysteinylglycine. The latter possibility cannot be overlooked since it has been reported that cystinyldiglycine produced a magenta or purple color in the Sullivan test (11, 12, 13). We have never observed any purple color in the tests carried out on the tissue incubation mixtures, but to rule out the possibility that any large quantity of cysteinylglycine was formed in the incubation mixtures the following experiments were carried out.

The product formed in a mixture of glutathione and enzyme preparation was isolated. Four hundred mg. of glutathione were added to 40 ml. of enzyme at pH 8.5. After 21 hours at 25°, the colorimetric determination, run on an aliquot of the mixture, indicated that 72 per cent of the cysteine of the glutathione, or 114 mg. of cysteine, had been liberated. The incubation mixture was treated with 1.0 ml. of 5.0 N H_2SO_4 and the tissue precipitate centrifuged off. The precipitate was washed two times with 10 ml. of 0.125 N H_2SO_4 and the washings united with the initial extract. The sulfur compounds were precipitated with 10 per cent $HgSO_4$ in 5 per cent H_2SO_4. The precipitate was removed by centrifugation, resuspended in water, treated with H_2S and the resultant solution centrifuged to remove HgS. The clear solution was aerated to remove H_2S and adjusted to neutrality with $Ba(OH)_2$. The $BaSO_4$ was removed by centrifugation. A trace of cupric salt was added and the solution was aerated until the nitroprusside test was negative. After standing overnight at 5°, 60.5 mg. of crystalline material separated. Microscopic examination of the crystals showed the typical hexagonal crystals of cystine. An additional 17 mg. of material separated after concentration of the solution to 10 ml. The 77.5 mg. of material isolated from the mixture showed 65.4 mg. cystine by the colorimetric determination, corresponding to 58 per cent of the cystine formed on incubation of the glutathione enzyme mixture. A solution containing 16.2 mg. of the material in 5.0 ml. of 1.0 N HCl gave a rotation of $-1.04°$ in a 2.0 dm. tube at 25° for the D line of sodium. $[\alpha]_D^{25} = -161°$ for the material isolated or $-190°$ on the basis of the cystine present. It seems clear that cystine was isolated and to show that cystinyldiglycine would have been detected had it been in our preparation the following experiment was done. Cystinyldiglycine was prepared[2] from glutathione by the procedure of Kendall, Mason, and McKenzie

[2] One hundred and sixty milligrams of glutathione were dissolved in 3.0 ml. of distilled water and placed in a constant temperature bath at 45° for 14 days. Mercuric sulfate in H_2SO_4 was then added to the mixture and the precipitate

(14). The product was tested by the cystine method, the light absorption of the color was measured in the Beckman spectrophotometer and compared with cystine and with a glutathione enzyme mixture. As Fig. 1 shows, the incubated sample of glutathione and enzyme preparation compares almost exactly with the cystine sample while the sample of cystinyldiglycine shows a different absorption. From this result we conclude that cysteinylglycine was not present to any appreciable extent in our enzyme experiments.

The liver of several species of animals showed a similar production of cysteine when tested under conditions the same as those used in the tests with guinea pig liver. The activities of homogenates of several animal livers based on guinea pig liver as 100 were: lamb 86, beef 59, human 50, pork 37, rabbit 17, and rat 2 to 5. The activity of rat liver was within the experimental error for the determination.

Properties of the Enzyme System Hydrolyzing Glutathione

Several properties of the enzyme were determined by use of the guinea pig liver homogenate. The activity of liver tissue was determined by use of a test in which the activity was expressed in terms of mg. cysteine (colorimetric) produced in 20 minutes from a mixture of glutathione and liver homogenate incubated at 25°. The mixture contained 4.0 mg. of glutathione and 2.0 ml. of 1:6 liver homogenate in a volume of 2.5 ml. of incubation mixture adjusted to pH 8.7 with acetate-veronal buffer. After deproteinization with metaphosphoric acid, the cysteine of the clear extract was oxidized and determined colorimetrically. The production of cysteine was proportional to the amount of enzyme preparation present (Fig. 2). The relationship of cysteine production to the concentration of glutathione with a given amount of enzyme is also shown in Fig. 2. The values for cysteine given on the curves are high because of the effect of glutathione in the cystine determination as discussed in the accompanying paper (6) but they demonstrate the relationships mentioned. The pH optimum of cysteine production by the liver homogenate was 8.7 for acetate-veronal buffer and 8.4 for glycine buffer (Fig. 3).

centrifuged off. The precipitate was decomposed with H_2S and the solution was aerated to remove H_2S. Barium hydroxide was added to give a pH of 7.0 and the $BaSO_4$ removed. The solution was evaporated at 45° to dryness and the dry material taken up in 6.5 ml. of water and adjusted to pH. 7.0. The aqueous solution was allowed to stand overnight at 0° and centrifuged. A 0.1 ml. aliquot was taken for use in the colorimetric test, it was diluted to 5.0 ml. with distilled water.

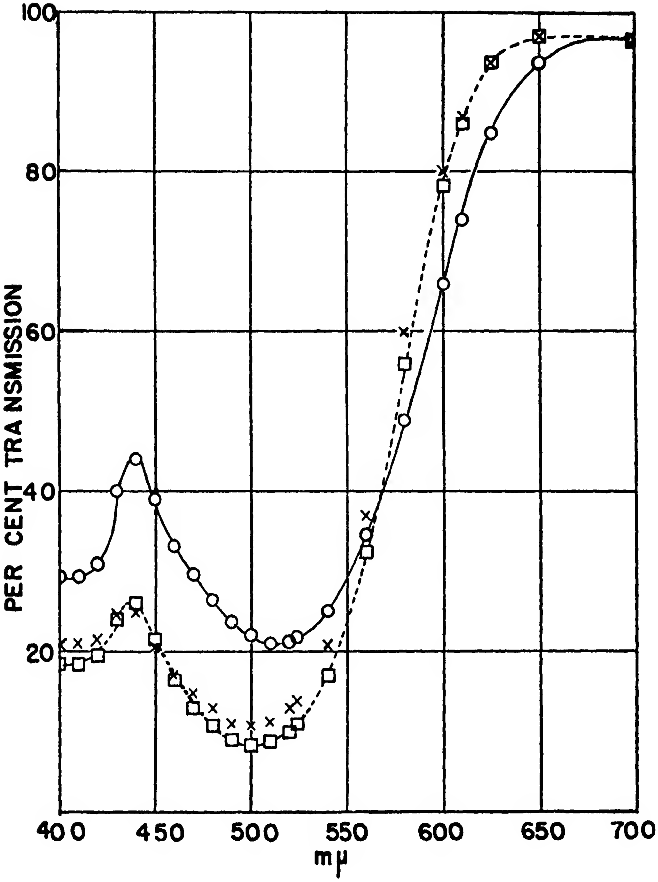

FIG. 1

Comparison of the Light Absorption of Cystine, Cystinyldiglycine, and Incubation Mixture of Glutathione and Guinea Pig Liver Preparation

Samples of cystine (□) (6.7×10^{-4} *M*), cystinyldiglycine (○) (8.0×10^{-4} *M* if the conversion of glutathione was 100 per cent), and glutathione-enzyme-mixture (X) were carried through the cystine colorimetric determination. The light absorption of the treated samples was determined in the Beckman spectrophotometer using a blank of distilled water as the standard. The concentration of cystinyldiglycine was calculated on the basis of complete hydrolysis of the glutathione used in its preparation. The concentration of glutathione in the glutathione-enzyme mixture sample as run in this test was 8.75×10^{-4} *M*. All concentrations are expressed in terms of the 5.0 ml. sample used in the colorimetric determination.

The concentration of glutathione in these tests was 2.0 mg. instead of the 4.0 mg. used in the test described above.

The activity of liver tissue homogenate (1:6) remained in the residue after centrifugation and crude material was prepared from the residue by washing it with

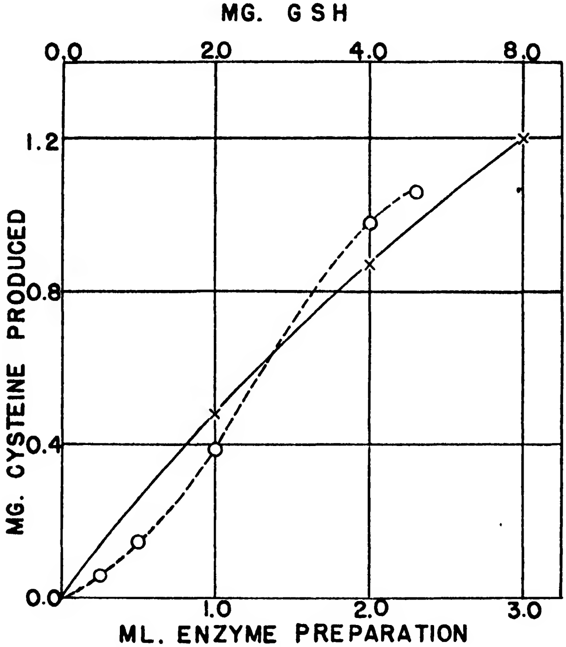

FIG. 2

The Production of Cysteine from Glutathione (GSH) by Guinea Pig Liver Homogenate

A mixture of liver homogenate (1:6), GSH and buffer to give a volume of 2.5 ml. at a pH of 8.7 was incubated for 20 minutes at 25°, deproteinized, and the cysteine of the clear extract determined colorimetrically. Curve X indicates the production of cysteine by 2.0 ml. of enzyme preparation in the presence of various amounts of GSH. Curve O indicates the production of cysteine by various amounts of the liver homogenate in the presence of 4.0 mg. of GSH.

50 per cent acetone-water solution at −5° and then with ether. The washed material was dried over sulfuric acid under reduced pressure. Active enzyme solutions were prepared by suspending 500 mg. of this product in 30 ml. of $M/30$ to $M/15$ phosphate buffer of pH 8.0 and centrifuging out the insoluble material. The activity of a sample was determined in the test described for the liver homogenate,

but the test was modified to the extent that 2.0 mg. of glutathione were used instead of 4.0 mg. and the time of incubation was extended from 20 minutes to 30 minutes. The solution prepared from the crude material contained only a small part of the total activity present originally in the tissue homogenate but it ap-

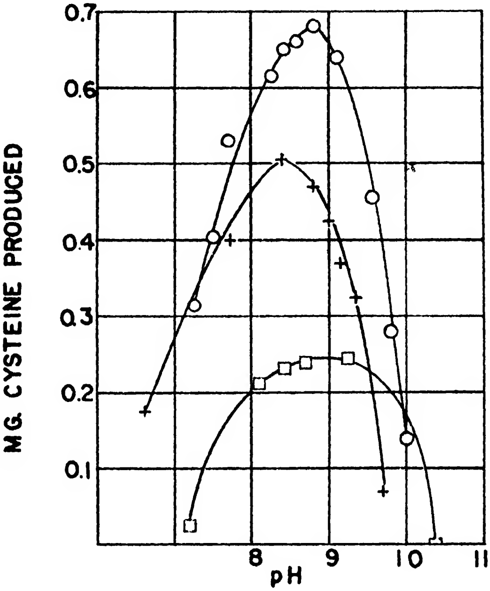

FIG. 3

Optimum pH of Cysteine Production

Production of cysteine by enzyme preparations as determined by the colorimetric method. The two upper curves indicate the production of cysteine by 2.0 ml. of guinea pig liver homogenate in 20 minutes at 25° in the presence of 2.0 mg. GSH in a total volume of 2.5 ml. The pH of the mixture was obtained with glycine buffer (X) and acetate-veronal buffer (O). The lower curve (□) indicates the production of cysteine by 2.0 ml. of partially purified enzyme preparation in 30 minutes, at 25° in the presence of 2.0 mg. GSH in a total volume of 2.5 ml. Acetate-veronal buffer was used to adjust the pH of the mixture.

peared to be homogeneous. The enzyme preparation behaved in much the same way as the liver homogenate with regard to optimum pH (Fig. 3) and also with regard to cysteine production with variation in substrate and enzyme concentration.

The enzyme preparation was most stable at a pH of 8.0 when kept at 25°. At this temperature it was completely inactivated at a pH of 3.7 in 105 minutes. It was also completely inactivated by exposure to 60° for 10 minutes while at pH 8.0.

The activity of the enzyme was apparently about the same under aerobic and anaerobic conditions although, of course, the iodine titration differed in most cases.

TABLE V

Reactivation of Dialyzed Enzyme

Material tested	Cystine produced in test *mg.*
Original enzyme solution	0.23
Enzyme solution dialyzed 20 hours	0.11
dialyzed 43 hours	0.09
dialyzed 89 hours	0.07
Enzyme solution dialyzed 43 hours	
plus dialyzate equivalent to original concentration	0.20
plus equivalent volume of heated enzyme solution	0.19
plus equivalent volume of 1:5 rat liver homogenate	0.18*
Enzyme solution dialyzed 89 hours	
plus dialyzate equivalent to original concentration	0.20
plus dialyzate equivalent to original concentration after irradiation 2.5 hours at pH 8.4 with Hg lamp	0.22
plus dialyzate equivalent to original concentration after heating 1.0 hour at 100° in 0.5 *M* HCl	0.18
plus dialyzate equivalent to original concentration after heating 1.0 hour at 100° in 1.0 *M* NaOH	0.17
plus dialyzate equivalent to original concentration after ignition	0.07

The enzyme solution was dialyzed against 20 volumes of distilled water at 0–5°. The 20 volumes of water were changed at intervals of 18–24 hours. At the end of the experiment the dialyzate was concentrated to small volume under reduced pressure at 45–50°.

* The activity of this sample was corrected for the activity of the homogenate added (0.03 mg.).

Dialysis of the enzyme preparation against distilled water or *M*/30 phosphate buffer of pH 8.0 at 5 to 10° resulted in inactivation of the non-dialyzable fraction (Table V). The dialyzates of two such experiments were concentrated at 45 to 50° under reduced pressure and tested for coenzyme activity. Addition of aliquots of the concentrated dialyzate to

the inactivated dialyzed solution restored a large part of the original activity of the preparation (Table V). Similarly, addition of an enzyme preparation that had been heated at 100° for 10 minutes also restored the activity. Interestingly enough, a preparation of rat liver that was practically inactive by itself also restored activity to the dialyzed preparation. The factor responsible for the activating effect of the concentrated dialyzate was very stable. Exposure, at pH 8.4, to the radiation of a mercury lamp (Westinghouse type A-H 4) for 2.5 hours at a distance of approximately 5 inches caused no decrease in the activating effect. Treatment of the concentrated dialyzate in either 0.5 *M* HCl or 1.0 *M* NaOH at 100° for 1.0 hour gave a loss of only 10–15 per cent, but ignition of the concentrate in the presence of a mixture of sulfuric and phosphoric acids completely destroyed the activating effect. The data are summarized in Table V.

Discussion

The data presented indicate clearly that guinea pig liver contains an enzyme that hydrolyzes glutathione with the production of cysteine. Probably the enzyme is the same as the one described by Schroeder, *et al.* (15) as antiglyoxalase and investigated in more detail recently (16, 9). For some reason guinea pig liver is particularly rich in the enzyme and rat liver is particularly poor in it, although it contains the coenzyme. From the fact that guinea pig liver normally contains very little cysteine, but does contain considerable amounts of glutathione it is clear that the equilibrium for the hydrolysis is pushed far to the glutathione side. In contrast, the liver preparations described above break down glutathione with the liberation of cysteine and the equilibrium is far to the amino acid side. It would seem to follow that in the intact liver the glutathione must be maintained as the result of coupling with some energy yielding reaction.

Summary

The reducing power of deproteinized guinea pig liver preparations toward iodine increases rapidly as the time between the killing of the animal and the addition of the deproteinizing agent increases. The increase is shown to be due to an enzymatic hydrolysis of glutathione with the liberation of free cysteine. The enzyme system is shown to be made up of a non-dialyzable enzyme and a dialyzable coenzyme.

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Effect of Light on the Reaction of Tyrosine in the Van Slyke Volumetric Amino Nitrogen Apparatus

Fraenkel-Conrat[1] has presented data to show that under conditions of intense artificial illumination, higher than theoretical amino nitrogen values (167 per cent) are obtained with tyrosine in the Van Slyke manometric amino nitrogen apparatus, both in the presence and absence of iodide. Inasmuch as theoretical values for tyrosine were obtained when the reaction chamber was completely darkened while deviations from theory became more pronounced as the intensity of illumination was increased, it was postulated that the higher values were due to the action of visible light upon the reaction mixture. In the volumetric apparatus the effect of light was observed by Fraenkel-Conrat to be "somewhat less pronounced" than in the manometric apparatus.

The existence of the phenomenon described by Fraenkel-Conrat means that analytical results for amino nitrogen, which are obtained under conditions where illumination of the reaction chamber is not avoided, may be in error if tyrosine, tyrosine derivatives, or other phenolic compounds are present in the samples analyzed. Furthermore, it is apparent that a simple correction cannot be applied in many instances since the results of Fraenkel-Conrat indicate that the magnitude of the error depends upon whether the tyrosine, for example, is present as the free amino acid or bound in peptide linkage.

For this reason, and because it is probable that the Van Slyke volumetric apparatus has been as widely used as the manometric apparatus for the determination of amino nitrogen, it was considered worthwhile to analyze tyrosine in the volumetric apparatus in the dark, under the conditions of illumination usually employed, and under the most intense conditions of artificial illumination described by Fraenkel-Conrat, to ascertain the magnitude of the tyrosine error in each case.

The results (see Table I) show that amino nitrogen values for tyrosine are obtained which are 100.6 per cent of theoretical when determinations are conducted in a photographic dark room, approximately 102 per cent of theoretical when conducted in the laboratory under ordinary conditions of illumination (diffused daylight), and approximately 106 per cent

[1] Fraenkel-Conrat, H., *J. Biol. Chem.* **148**, 453 (1943).

of theoretical when conducted under conditions of intense illumination (100-watt-spotlight-bulb held as close to the reaction chamber as possible without interfering with the mechanical shaking of the vessel). Potassium iodide does not influence the extent of the reaction under any of the conditions employed.

The effect of light on the tyrosine-nitrous-acid reaction in the volumetric apparatus is significant, but it is extremely small as compared with the effect found by Fraenkel-Conrat with the manometric apparatus. Moreover, it is evident that the error due to the action of diffused daylight on the tyrosine-nitrous-acid reaction mixture in the volumetric apparatus is so slight (less than 2 per cent) that it may be considered as negligible in the routine analysis of protein hydrolyzates where only a fraction of the total amino nitrogen is present as tyrosine.

TABLE I

The Analysis of l-(−)-Tyrosine in the Van Slyke Volumetric Apparatus Under Various Conditions of Illumination

Averages of two or more analyses; macro apparatus; 15 minute reaction period; temperature 24.5 to 26°C.; 8 mg. of tyrosine, (N = 7.704) equivalent to 0.6185 mg. of amino nitrogen per ml.

Conditions	Amino Nitrogen Found	Amino Nitrogen
	mg. per ml.	*per cent of theory*
Dark, KI absent	0.622	100.6
Dark, KI present*	0.622	100.6
Diffused daylight, KI absent	0.629	101.7
Diffused daylight, KI present	0.630	101.9
Diffused daylight plus 100-watt-spotlight-bulb, KI absent	0.655	105.9
Diffused daylight plus 100-watt-spotlight-bulb, KI present	0.654	105.7

* 0.5 per cent potassium iodide added with nitrite solution.

It seems likely that factors in addition to light intensity must be considered in explaining the extremely high Van Slyke manometric values for tyrosine reported by Fraenkel-Conrat.

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GEORGE W. IRVING, JR.

March, 1944

BOOK REVIEWS

The Chemistry of the Amino Acids and Proteins, with Addendum, pages 1033–1290 Inclusive of Some of the Advances Since 1937. Edited by CARL L. A. SCHMIDT, M.S., Ph.D., Professor of Biochemistry and Dean of the College of Pharmacy, University of California. Second Edition. Charles C. Thomas, 220 East Monroe St., Springfield, Ill., 1944. xxvii + 1290 pp. Illustrated. Price $10.00.

The rapid progress in the field of protein chemistry can scarcely be better illustrated than by the fact that it has seemed desirable to issue a new edition of this important handbook after the expiration of only six years. The Editor with his associates have departed somewhat from custom however. At the suggestion of the publisher, instead of attempting to rewrite the eighteen chapters, with their numerous subordinate sections and subsections, additions to the material presented in the first edition have been prepared, in most cases by the original contributors, which together constitute pages 1033 to 1290 of the present volume. Separate author and subject indices have been provided. A special note on the wrapper indicates that the addendum is available separately for the benefit of those who already possess the original edition.

It is stated in the introduction that no attempt has been made to cover the literature completely. Nevertheless, the new sections furnish an excellent and reasonably complete review of the protein chemistry of the past few years, a part at least of the literature that appeared in 1942 having been included in most of them.

Schmidt's handbook as a whole fulfills an extremely useful function. Although many of the chapters are perhaps too comprehensive and detailed for the beginning student, one who is more advanced may have some confidence that he will obtain a clear outline as well as a key to the basic literature of most of the topics in which he may be interested. The segregation of the newer material may well prove a convenience to many users.

Inasmuch as the interests of the Editor and of most of the contributors are in the physical properties of proteins, greater emphasis is placed upon the behavior of proteins than upon proteins themselves as organic chemical substances that occur in nature as essential components of the living cells of all tissues as well as of secretions and exudates. This is perhaps an expression of the trend of the times. It would be difficult to deny that the contributions of physical chemists to fundamental protein chemistry during the past two decades have overshadowed those of the physiologists and organic biochemists, and protein chemistry at the present time bids fair to become a discipline in which the equations of mathematics rather than those of organic chemistry play the dominant role. The truth is that although we know a great deal about proteins, we still do not know what they are: the contents of the present book illustrate this point vividly.

The book suffers from the defect common to most attempts to combine the work of different authors into a single volume, namely a difference in point of view and style of presentation on the part of the several contributors. Furthermore not all of the chapters are equally adequate as treatments of the material discussed. However, the rate of progress being what it is today, so ambitious a project as the description of the whole of protein chemistry in one volume, if anything but a superficial statement were to be aimed at, would be a hopeless undertaking for a single author. Teachers and investigators alike have reason to be grateful to the Editor and his collaborators for their self-sacrificing labors. In view of the wealth of material that has been included, such obvious omissions as a discussion of the virus proteins, or an adequate description of the preparation and properties of the proteins of plant seeds, or of the enzyme proteins, may perhaps be overlooked. One may be permitted to hope that, if still another edition is required at a later date, these points will be considered and that a more detailed and comprehensive subject index will be prepared.

H. B. VICKERY, New Haven, Conn.

Vegetable Fats and Oils. By GEORGE S. JAMIESON, United States Department of Agriculture. Reinhold Publishing Corporation, New York, N. Y., 1943. 508 pp. Price $6.75.

The eleven years since Dr. Jamieson's book appeared in its first edition has been a most active period of investigation and technological advance in the field of vegetable fats and oils. During the entire period the author has been in a position to keep in touch with other details than those with which he has himself been engaged. The breadth of his knowledge and interest is apparent throughout the authoritative writing of his book.

In general, the arrangement of the second edition follows the outline of the first but new material has been added and numerous editorial changes were made to clarify earlier vague and misleading sentences. Very few serious errors have been found in the second edition. An obvious one occurs on p. 346 where one of the few structural formulas (elaidic acid) was misprinted. Calculated molecular weights cannot be checked in many instances. Typographical errors are especially noticeable in Chapter V on the fatty acids. Interestingly, some of them were carried over from the first edition.

New material was added in several ways: (a) insertion of phrases, sentences, or paragraphs into the original text, (b) extension of the literature references dispersed freely throughout the book, and, in a few cases, (c) complete rewriting (e.g. oleic acid, p. 345). However, the reviewer has the opinion that this treatment of newer findings leaves much to be desired. In connection with oleic acid, no mention was made either in the text or in the added references of the important contributions of J. B. Brown and others to the purification of oleic acid by crystallization procedures—despite the fact that this technique has been the most acceptable procedure for several years. On p. 378, the author discusses the double melting of fats indicating that no explanation of the phenomena is known. The fact is that sufficient information on the polymorphism of glycerides is known to provide at least a qualitative conception. On p. 420, the discussion on conjugated double bonds carries no reference to the extensive use of spectroscopic procedures.

For the purpose intended, Dr. Jamieson's book has always been eminently satisfactory and the second edition will be useful to those who own the first edition. It is an invaluable reference book for more than 300 vegetable fats and oils and the methods used in their preparation, analysis and handling in manufacturing operations. At the end of the book there are 19 tables of data and names of botanical families and species. The reviewer is in complete agreement with the author's comment (p. 228), "It is very important in future studies that the botanical species should be known from which the oil was obtained; otherwise the oil should not be studied." It would have been desirable to place this sentence more prominently for the emphasis.

HERBERT E. LONGENECKER, Pittsburgh, Pa.

Frontiers in Cytochemistry. The physical and chemical organization of the cytoplasm. Edited by NORMAND L. HOERR. Biological Symposia, Vol. X, 1943, Jacques Cattell Press, Lancaster, Pa. $3.50.

Frontiers in Cytochemistry is the published record of a symposium on "the physical and chemical organization of cytoplasm" held in honor of Dr. R. R. Bensley, Professor Emeritus of Anatomy of the University of Chicago on his seventy-fifth birthday. The character of the volume is best shown by a list of the subjects discussed:

Foreword. NORMAND L. HOERR

In Appreciation of Professor R. R. Bensley. E. V. COWDRY

The Chemical Structure of Cytoplasm as Investigated in Professor Bensley's Laboratory during the Past Ten Years. ARNOLD LAZAROW

Some Considerations on the Application of Biological Oxidation-Reduction Reaction Systems to the Study of Cellular Respiration. E. S. GUZMAN BARRON

Ultracentrifugal Studies on Cytoplasmic Components and Inclusions. H. W. BEAMS

Electrolytic Solutions Compatible with the Maintenance of Protoplasmic Structures. ROBERT CHAMBERS

Distribution of Nucleic Acids in the Cell and the Morphological Constitution of Cytoplasm. ALBERT CLAUDE

Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice. E. V. COWDRY

Histochemical Analysis of Changes in Rhesus Motoneurons after Root Section. ISIDORE GERSH AND DAVID BODIAN

Methods of Isolation of Morphological Constituents of the Liver Cell. NORMAND L. HOERR

Electrolytes in the Cytoplasm. OLIVER H. LOWRY

Fibrous Nucleoproteins of Chromatin. A. E. MIRSKY AND A. W. POLLISTER

The Ultrastructure of Protoplasmic Fibrils. FRANCIS O. SCHMITT, CECIL E. HALL, AND MARIE A. JAKUS

Mineral Distribution in the Cytoplasm. GORDON H. SCOTT

Studies on Macromolecular Particles Endowed with Specific Biological Activity. KURT G. STERN

The Chemistry of Cytoplasm. R. R. BENSLEY

The papers in the volume on Frontiers in Cytoplasm, adhere in the main, to the subtitle, the organization of protoplasm. One important property of living matter is kept constantly to the fore, namely, the continuity in the structure of protoplasm. It is interesting to have watched during more than a quarter of a century of research on protoplasm, the gradual change from what might be termed a solution hypothesis of protoplasm to one in which the structural units are regarded as forming a continuous net. Earlier concepts on the structure of living matter were based on its fluidity, its supposed miscibility in water, its suspension of granules, and its emulsion of fat. Today these properties are all either discarded or relegated to secondary roles. In this transition of thought, Dr. Bensley has played a major part.

In the first chapter of the volume Lazarow lays a chemical foundation by distinguishing two major constituents of liver, a "glycogen particulate" and a lipoprotein complex. He concludes that the structural organization of a cell is due to the orientation of asymmetric micelles. In the third chapter Beams interprets a structure in which it is possible to stratify mitochondria with high centrifugal forces and yet permit the presence of an ultramicroscopic framework. Claude presents a chemical study of secretory granules, mitochondria, and microsomes, which he finds are closely related but not identical constituents of protoplasm. He lays emphasis on the physiological importance of these phospholipid and ribose nucleoprotein particles, and points out that their important role does not detract from the presence or role of the framework of nucleoproteins, but, on the contrary, the latter may be the seat of origin of the former.

The work of Dr. Bensley culminates in his discovery of plasmosin. The chapters in the commemoratory volume rise to a climax in the discussion of plasmosin. Hoerr describes the discovery of plasmosin and ellipsin, the former a liver extract, a gel and fiber producing protein, the latter an insoluble residue upon which the integrity of the cell as a unit of structure depends. Dr. Bensley regards ellipsin as a complex of proteins.

There is some question as to the place where plasmosin occurs in greater abundance. Dr. Bensley says, "I would not wish to reject the idea that the nuclei contain plasmosin but . . . the rapid extraction of plasmosin from . . . cells without much loss of nuclear protein inclines me to the opinion. . . that it is in large measure a constituent of cytoplasm." Mirsky and Pollister say, " . . . plasmosin is a nucleoprotein . . . located largely, if not entirely, in the nucleus of the cell . . . ."

Several chapters in the book are excellent treatises of subjects of great significance to the physiology of the cell, but bear less directly on structure and as my space is limited I shall not attempt to review them all, but conclude with a brief discussion of Dr. Bensley's work.

The volume is closed with a chapter by Dr. Bensley on the chemical structure of cytoplasm. For my own satisfaction I should like to reword, somewhat in accordance with Dr. Bensley's suggestion, the two statements by K. H. Meyer which Dr. Bensley says will then be acceptable to the majority of students of cell structure.—Certain of the structural units of protoplasm are linear molecules joined into a network of primary valence chains held together by chemical bridges which are tied by molecular adhesions of the nature of residual valences or hydrogen bonds.

As I read the pages of this book, I am deeply impressed by the fact that Dr. Bensley and his coworkers have been so earnestly at work in so difficult a field, which has been the subject of so much controversy. I need not think far back to recall more than one instance where I have been told that any speculation on protoplasmic structure is utterly futile when so little is known of the structure of relatively simple systems such as the proteins. It is the courage as well as the keen insight and ability of a Bensley that advances science in the face of opposition by the over cautious pessimists who fear the Frontiers. It is a pleasure to recall those chemists and physiologists who have joined the biologists with so much good will and sincere interest in their search for the basic structure of living matter; to name but a few of them: Freundlich, Astbury, Szent-Györgyi, and Pauling.

Dr. Bensley closes the volume of Frontiers in Cytochemistry with some fundamental ideas. He says, plasmosin is the substance associated with gelation in protoplasm, whereas ellipsin is concerned with the more stable structural substrate of the cell. And again, cytoplasm has no ultimate structural unit but consists of several, perhaps many, different types of units, all cooperating in an orderly fashion to produce that ensemble of properties which we call life.

Wm. Seifriz, Philadelphia, Pa.

Laboratory Manual of Spot Tests. By Fritz Feigl, Laboratorio da Producao Mineral, Brazil. Translated from the German by Ralph E. Oesper, Professor of Chemistry in the University of Cincinnati. Academic Press Inc., New York, N. Y., 1943. Xii + 276 pp. Price $3.90.

This contribution to the literature on spot test analysis deals with techniques and the physical and chemical aspects of the diverse reactions that culminate in observations of analytical significance. While dealing with the subject matter presented in his earlier books "Qualitative Analysis by Spot Tests" and "Specific and Special Reactions," the Manual differs considerably in scope and contents from these well known works. The book considers the analytical reaction from the viewpoints of sensitivity and reliability and discusses such factors, influencing drop reactions, as the threshold values of particle size and color intensity that must be reached before a material reaction can be discerned by the eye. The presentation of these physico-chemical aspects of analytical reactions is thorough and will prove of value to the advanced student and research worker in the field of analytical chemistry.

A considerable portion of the contents represents the recent work of Feigl and his collaborators that has been published in American journals. The Laboratory Manual does not contain a bibliography. This omission detracts from the serviceability of the volume. The book contains considerable information of value in the field of Industrial Hygiene. Of particular interest are the novel staining methods for the detection of amorphous silica and its differentiation from crystalline silica by the demasking of silver-ammine chromate, and the detection of insoluble basic materials in silicates by means of an equilibrium solution of nickel dimethylglyoxime. The method for the detection of sulfur dioxide in the presence of other volatile sulfur compounds by the induced oxidation of nickel hydroxide is, likewise, of interest in the study of atmospheric pollution.

The Manual will prove useful to chemists engaged in the analysis of the newer

explosives and diverse organic compounds brought to their attention as a result of war technology. A clue to their identity may often be secured by the application of the tests for the more common organic groupings, for example nitroso, amino, hydroxy, aldehyde, ketone, phenol, mercaptan, sulfonic and carboxylic acids described in the book.

Of particular interest is a chapter devoted to quantitative methods of spot test analysis. In the majority of problems confronting the analytical chemist the identity of the constituents is known from the history of the sample, and the usual request is for an assay of one or more stipulated components. The methods of drop colorimetry are useful as a preliminary means for gaging the order of magnitude of a principal component, for the estimation of traces, and as a means of analysis in the case of minute samples. The simplicity and elegance of qualitative drop reactions may mislead some readers into the belief that the quantitative aspects of the technique presents a royal road to analytical chemistry. Such is not the case; reliable results can only be obtained by exercise of the usual care and effort that must of necessity be devoted to all quantitative analytical work.

The Manual is more than a compilation of novel analytical methods and microchemical techniques. To those familiar with Dr. Feigl's trials since the loss of his laboratory at the University of Vienna and the renewal of his activities in Brazil the book is an expression of an indomitable spirit and a courageous mind.

HERMAN YAGODA, Bethesda, Md.

Semimicro Quantitative Organic Analysis by the late E. P. CLARK, U.S. Dept. of Agriculture. The Academic Press, Inc., New York, N. Y. V + 135 PP., with original drawings and tables. 1943, $2.50.

The obvious advantages of speed and economy of material associated with the Pregl methods of organic analysis have led to their wide adoption, although the analyst must always bear in mind that in almost every detail of procedure he is working up against the extreme limits not only of the accuracy of his apparatus but also of his own skill and patience. Besides, it frequently happens that he is not restricted to real "micro" samples (say up to 5 mg.) since plenty of material is often available, and it may be next to impossible for one reason or another to procure a real "micro" balance.

Under such conditions this book of Dr. Clark's will be found very useful. He describes in all necessary detail certain common determinations where samples of 10–25 mg. are used, together with carefully drawn and clearly reproduced drawings of the apparatus required. Much of his apparatus is nearly enough identical with that recommended by the Standardization Committee of the Division of Analytical and Micro Analysis (American Chemical Society) to permit the use of "standard" parts, but unfortunately no reference is made to the publications of this Committee, so that if the prospective user is asked about this by his supply dealer, either one of them will have to figure out the answer himself.

Dr. Clark recommends a certain "semi-micro" balance of domestic manufacture, but gives sufficient reference to the literature so that the reader may judge if perhaps one of his own regular analytical balances may not be suitable. This involves a determination of the precision of the balance, and here the reader may be permitted a doubt as to whether the author had drawn for himself sufficiently clear

distinctions between the concepts of accuracy, precision, and sensitivity as applied to a chemical balance.

Dr. Clark gives few critical evaluations of procedures differing from those recommended and few literature references, so that if the user should run into difficulties the book itself will not be of much help in "troub'e-shooting." It is therefore better adapted for use by the experienced analyst—it would be less suitable for beginners. The directions for all the procedures however are given in considerable detail, and those who knew the thorough and painstaking work of Dr. Clark will easily take his word for it (p. IV) that these are "simple working well tested methods, which can be followed to a successful conclusion."

One unusual feature is a thorough discussion of the calculation of empirical formulas from analytical data, together with an ingenious table to facilitate such calculation. In the little table of gravimetric factors (p. 92), that for CO_2 is given incorrectly—it should be 0.2929, log 43599.

The book should make a valuable addition to the working library of any chemist interested in Organic analysis.

FRANCIS W. POWER, S. J., New York, N. Y.

Plant Viruses and Virus Diseases, second revised edition, BY F. C. BAWDEN, Head, Plant Pathology Department, Rothamsted Experiment Station. Chronica Botanica Company, Waltham, Mass.; G. E. Stechert and Co., New York City. 1943, xi + 294 pp., 55 figures, Price $4.75.

This book is a completely revised edition of Bawden's "Plant Viruses and Virus Diseases" published in Holland in 1939 by the same firm. Many of the chapters have been rewritten and all have been modified. Nevertheless, the book follows closely the pattern established in the first edition. The presentation of material has been rearranged slightly so that the new edition has sixteen chapters instead of fifteen. The chapter headings are introductory survey; symptomatology (2 chapters); transmission; relationships between viruses and their insect vectors; virus strains, mutation, and acquired immunity; serological reactions of plant viruses; methods of purification; properties of purified virus preparations; optical properties of purified virus preparations; inactivation of viruses; the sizes of virus particles; physiology of virus-diseased plants; the classification of viruses; the control of virus diseases; and discussion on the origin and multiplication of viruses. The book was written partly because the first edition is out of print and the type was lost during the invasion of the Netherlands, and partly in order to bring the literature on plant viruses up to date. Because of restricted communications, the author achieved only limited success with respect to the second purpose, for although the manuscript was completed in the autumn of 1942, many of the papers published in 1942 and some of those published in 1941 were apparently not available.

This book has many excellent features. It is written in a clear, readable style and is amply illustrated with figures and tables. In spite of war-time restrictions, the publishers succeeded in producing a truly handsome book, remarkably free of printer's errors. The subject matter covers a wide range, and many phases of it are excellently treated. The reviewer was particularly impressed by the thought provoking discussion of the origin and multiplication of viruses, even though he is

not inclined to accept all of the author's conclusions. The discussion of classifications of viruses, in which the advantages of schemes based upon the intrinsic properties of viruses, such as Chester's serological relationships and the cross protection reactions, are advocated, seems to the reviewer to be very constructive. The author is to be complimented for having tested in his own laboratory certain important claims made by other investigators before presenting them to his readers.

Unfortunately, however, the book is not without significant limitations, limitations of a sort that escape the attention of the casual reader, but which are a source of dismay to one more familiar with this subject. It contains more than the usual bias expected of any author writing in a field in which he has made important personal contributions. As an example, more than eight pages are devoted to the question of the crystal structure of the viruses, a subject investigated principally by workers close to the author, but only about half a page is devoted to the important and elsewhere widely investigated question as to whether these crystalline proteins are actually viruses. In some instances, universally available literature containing interesting and pertinent data is ignored entirely. The reviewer was able to detect seven instances in the field of biochemistry alone. In each case, the paper in question was abstracted in a British journal received in this country before the autumn of 1942. Attention to bibliographic detail is not always meticulous, for occasionally the author appears to assign credit for certain observations improperly, even though the record in the literature is not ambiguous. For example, in the discussion on page 85, it is not made at all clear that Black was the first to deal effectively with the question of plant virus inhibitors in insect vectors.

The subject matter of this book touches a wide variety of scientific disciplines, from systematic biology on the one extreme to hydrodynamic theory on the other. It is a tribute to the author that he is able to present intelligible discussions of so great a diversity of topics. However, it is virtually impossible for anyone to be thoroughly competent in all of these fields, and the book does suffer from numerous inadequacies which can be attributed to this cause. The treatment afforded many topics is not critical in the true sense of the word. For example, because of the seemingly complete acceptance of an unsubstantiated opinion which found its way into the virus literature, the author was led to make as his own the following statement: "The mathematical difficulties in deriving an equation for the sedimentation of non-spherical particles are so great that it has not yet been done." (p. 220). This statement is contrary to fact, for since 1936 an acceptable mathematical analysis of the frictional resistance to translation as related to the dimensions of both rod-like and plate-like randomly oriented ellipsoids of revolution has been available. This equation is valid for boundary conditions not unlike those of the usual form of Stokes law for spheres. It is only a question of elementary algebra to express this result as a sedimentation equation. Other errors of fact have been committed. For example, it is stated in connection with the discussion of the "layering phenomenon" shown by tobacco mosaic virus that, "Other fluids containing rods, for example, solutions of vanadium pentoxide, form similar tactoids, but with these the separation of a liquid crystalline layer has not been observed." (p. 179). On the contrary, an identical separation was described for

vanadium pentoxide solutions in 1927, and attention was called to this work in the literature on tobacco mosaic virus as early as 1939.

It would be a mistake to overemphasize the importance of these and of other limitations that could be cited. However, they cannot be passed over too lightly, for their presence detracts from the value of a book which is otherwise remarkably good. This is particularly true because most readers who are not thoroughly familiar with the subject matter are apt not to recognize the errors and might, therefore, be misled by them.

About one-third of the space of the book is devoted to the discussion of subjects which fall within the scope of biochemistry and physical biochemistry. This seems to the reviewer to represent a reasonable estimate of the importance of the chemical approach in the field of plant viruses. Accordingly, the author is certainly justified in limiting the treatment of this subject in a book of general character. Nevertheless, a thorough treatment of all of the phases of the chemistry and physics of plant viruses would require considerably more space. This work probably was not intended to fulfill such a purpose.

Mr. Bawden has performed a real service to science in assembling and coordinating widely diversified observations scattered throughout many journals serving numerous specialties. On the whole, this book is one which the reviewer can recommend heartily to students of science who would like to have a concise and readable source of information concerning important new developments with respect to plant viruses and plant virus diseases.

MAX A. LAUFFER, Princeton, N. J.

The Theory and Practice of Semimicro Qualitative Analysis. BY G. B. HEISIG. (331 + xiii pages) W. B. Saunders Co., Philadelphia, Pa.

This text has been written essentially in terms of the standard classical procedure for qualitative analysis of the ions, reducing the quantities of the substances to be determined and the reagents for their determination proportionately until relatively small amounts are employed Apparatus is thus reduced in size, and time, equipment and material conserved.

The "Suggestions to Instructors" are well thought out, clearly put and adaptable to any type of course—macro as well as micro. The "Introduction" states the scope of the text and lists in a logical manner the ions to be detected. The number of anions mentioned are more than are usually considered in an elementary course in Qualitative Analysis.

The theoretical portion of the book, Chapters I to VII, inclusive, treats in detail of the theoretical considerations which must be covered in teaching such a course to beginners. It would seem, however, with such great stress laid on the Debye-Hückel, Brønsted, Lewis and Werner concepts, that further use might well be made of organic reagents, both as separatory and confirmatory agents. The summary of the modern theory of ionization is very good, as are the problems and exercises at the end of each chapter.

In discussing the derivation of the water constant on page 39, the numerator in the first equation should appear as a squared term in accordance with the Mass Action Law. The explanation of precipitation and solution of difficultly soluble

substances is excellent. It would seem, however, that the term "colloidal solution" is a misnomer from the definitions usually given for both a solution and a colloid. It would also seem that with so much stress laid on the Brønsted theory and the activity concept, that a greater amount of space might have been devoted to a development of the theoretical aspects on the spatial arrangement of the coordinated groups in the Werner compounds.

The sections on hydrolysis, buffers, amphiprotic bodies and activities are exceptionally clear and well written. This is particularly true of the application of the activity concept to ionization, solubility product and hydrolysis constants.

The practical, or experimental, portion of the book is subdivided into a consideration of procedures for the separation and detection of the cations and anions, to each of which has been assigned 6 chapters, and 1 chapter on systematic analytic procedures, including a few of the commoner dry reactions.

The methods employed for determination of the cations are, essentially, standard. It might be argued that the determination of sodium ion by zinc uranyl acetate could lead to erroneous results when the sample to be analyzed has been too long in contact with glass. The reviewer has had such unhappy experiences with samples supposedly sodium-free after their solutions had been allowed to stand for one to two weeks, although the freshly prepared solution gave no reaction for sodium.

The procedures for the anions are based on the work done by the author's former students. These call for the use of strontium and cadmium acetates in addition to the usual calcium, barium and silver salts, and the substitution of nickel for zinc. As is usually the case, several of the anions may appear in more than one place in the scheme, owing to their reactivities at various dilutions. This may lead to some confusion, particularly with a beginning analyst.

The book is well bound and free from typographical errors, the main exception to the latter statement appearing on page 263. It is a readable text and should be a welcome addition to the literature on Qualitative Analysis.

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